Use of 1,2:5,6-Dianhydrogalactitol in Studies on Cell Kinetics-directed Chemotherapy Schedules in Human Tumors in Vivo


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ABSTRACT

Recently, it has been shown that 1,2:5,6-dianhydrogalactitol (DAG) can cause reversible alterations in cell cycle kinetics. Following treatment of CHO cells in vitro and Ehrlich ascites tumor cells in vivo, significant increases in the fraction of cells in S phase were observed to occur, and this was followed by an increase in the fractions of cells in G2 and mitosis. Treatments with S or G2-M phase-specific drugs at the peak enrichment times after DAG was given resulted in greater cell kills than when given by any other schedule.

We have extended these kinetics-directed drug schedule studies to human tumors in vivo. The first phase was to determine whether DAG could be used to perturb cell kinetics in vivo as effectively in patients as it was in vitro. In 14 of 17 tumors studied, increases in the S-phase fractions were observed (ranging from 30 to 240% increases). The hr at which the S-phase peaks were observed (post-DAG treatment) was variable among the patients and among the tumors studied. However, this points out the value of obtaining actual cell kinetics data from serially biopsied tumors growing on the body surface and illustrates the importance that these data may have in helping to select an optimal time at which to give an S phase-specific drug. If such tumor cell kinetics-directed scheduling is ultimately shown to be effective, it will represent a means of individualizing therapy for a large fraction of tumor patients whose tumors are growing on or near the surface of the body. The tumors utilized in these studies were squamous carcinomas of the head and neck, skin, anus, and cervix; adenocarcinomas of the breast and rectum; and malignant melanoma.

The second phase of this study will be to determine the tumor responses in patients treated with such kinetics-directed schedules.

INTRODUCTION

Since most large solid tumors contain only small fractions of cells in the cell cycle (11, 18), the cycle-active drugs will be minimally effective. For example, a tumor with an 8 or 10% fraction of cells in S phase will not be very sensitive to S-phase-specific agents such as hydroxyurea or ara-C.3 However, if the fraction of cells in S phase can be enriched, the tumor might become more sensitive to cell cycle phase-specific anticancer drugs, especially if the time at which enrichment occurs in S phase can be determined. In patients whose tumors are growing on or near the body surface, this may be achieved through the study of tumor cell "kinetics-directed treatment schedules." By kinetics-directed treatment schedules, we mean that the patient's own tumor growth kinetics properties would actually be studied and used to direct the timing of his drug treatments. For example, Drug A is given at 0 hr to perturb the tumor cytokinetics. Biopsies taken before and at various times after treatment with Drug A are studied to determine the relative changes in the percentage of cells in the various phases of the cell cycle. Through the use of multiple biopsies and the associated relative changes in tumor cytokinetics, near optimal treatment schedules may be determined, the rationale, therefore, being that the first drug is used to cause enrichment of a fraction of cells into S (or another) phase, where the second drug kills them most effectively. In contrast, a "kinetics-based treatment schedule" is one in which the cytokinetics effects and the timing for drug administration are determined from historical and empirical data rather than from direct measurements made on that patient's own tumor.

Several in vitro and in vivo studies have been reported on kinetics-directed treatment schedules, both in human and other tumors. The enriching or synchronizing drugs have included hydroxyurea, ara-C, vincristine, BLEO, DAG, and others (1-7, 9, 10, 15, 19).

Recently, we reported successful in vitro kinetics-directed treatment schedules (2). Ten hr after treatment of Chinese hamster ovary cells with DAG, a 160% enrichment of cells into the S phase of the cell cycle was observed. Treatment at that time with the S-phase agent, ara-C, caused a greater cell kill than when given at any other time schedule. Eighteen hr after an exposure to DAG, the cells exhibited a 220% increase in the fraction of cells in the G2-M phases of the cell cycle. BLEO, known to be most effective against G2-M cells, had the greatest effect on cell killing when administered at that time. Rapid analyses by FMF techniques were used to determine the DAG-induced kinetics changes, thus allowing treatment with the second drugs at the most opportune time. The DAG-induced kinetics changes were also demonstrated in a line of human adenocarcinoma of the stomach in vitro and in Ehrlich ascites tumor cells in vivo. In all cases, the enrichment of cells into S phase was reversible and was followed by a reversible blockade in G2-M (2).

We have now extended these DAG studies to human tumors in vivo, and that is the subject of this report. Patients whose tumors were growing on or near the body surface were treated with DAG. Serial biopsies of tumor (and normal) tissues, ob-
tained before and at 6- to 12-hr intervals after DAG, were assayed for DAG-induced cell kinetics changes using FMF techniques.

MATERIALS AND METHODS

Clinical Procedures. Patients selected for these studies were those who were able to give informed consent and whose tumors were readily accessible for biopsy. A complete physical examination was obtained and recorded. Precise location of any tumor, as well as photographs and measurements of tumor size and 2 perpendicular diameters, were recorded. A bone marrow biopsy, routine hematological studies, and blood chemistries for liver and renal functions were obtained, as were X-rays and other studies appropriate for the particular tumor type being treated.

The tumors studied were: 3 squamous carcinomas of the head and neck, one malignant melanoma, 3 squamous carcinomas of the anus, one adenocarcinoma of the rectum, 6 squamous carcinomas of the cervix, one breast carcinoma, and 2 squamous carcinomas of the skin.

A separate group of cancer patients was injected initially, each with a single dose of DAG to determine a minimum dose which would cause cell kinetics perturbations in the tumors. Patients given single doses of 10 or 50 mg/sq m exhibited no changes in tumor cell kinetics patterns. However, a single dose of 100 mg DAG per sq m did alter tumor cell kinetics (as observed via FMF analysis) and was used on the 17 tumors in the study reported here.

Biopsy Sample Preparation for FMF Analysis. The term "degree of enrichment" used in this paper is defined as the relative increase in the fraction of cells in S phase (after DAG treatment) as compared to pretreatment or baseline kinetics values. The tumors studied were usually 5 cm or smaller in the longest dimension. Depending on the size of the tumor, multiple biopsy samples were obtained at each sample time, before and at 6- to 12-hr intervals after the drug synchrony treatment, and the cytokeratin data were used to determine: (a) the degree of enrichment into S phase; and (b) the time at which the highest degree of enrichment was measured. It takes less than 1 hr (from biopsy) to determine cell kinetics changes by the methods described herein. Appropriate normal tissue samples were obtained from all patients at each sample time to determine the relative effects of DAG on normal cell kinetics.

Processing of tumor biopsy was started within 10 min of the time biopsies were taken. The 0.5- to 1-cm sample was minced with scissors and sonicated to pieces less than 1 cu mm in size, transferred to a small flask, and agitated for 10 min in the presence of 0.5% pepsin (Accurate Co., Hicksville, N. Y.) via the method of Zante and Schumann (22). The samples were processed until a maximum of 10,000 cells was removed to a beaker, and an equal volume of 0.1% RNase solution (Sigma Chemical Co., St. Louis, Mo.) was added. The cells were stained at room temperature for 20 min with ethidium bromide:mithramycin (Sigma) (22) and analyzed on a Coulter TPS-1 FMF instrument with the laser tuned to 488 nm.

The samples were processed until a maximum of 10,000 cells was reached in the G1 peak channel of the DNA histogram. The G2 peak was set to Channel 30, and the G2-M peak occurred approximately in Channel 60. (The linearity of the FMF instrument is checked routinely and has been found to range from 1.97 to 2.1; i.e., the G2-M peak channel occurs at approximately 2 times that of the G1 channel.) Using this technique, we generally obtain histograms whose coefficients of variation range from 4 to 6 for solid tumor samples and 2 to 4 for tissue culture samples. Companion biopsy samples of normal cells were processed in the same manner and were used as a normal standard and to locate the G1 channel of the aneuploid tumor samples. Each sample type was processed both separately and mixed together, while the FMF instrument was maintained at identical settings.

RESULTS

General Comments. Since no parallel untreated tumor controls can be run for patient cell kinetics studies such as these, pretreatment biopsies were obtained and processed for FMF analyses and used as untreated baseline controls. The fractions of cells in G1, S, and G2-M were calculated from the DNA FMF histograms, and all other DAG-induced kinetics changes in that patient were compared to them. As often as possible, more than one biopsy was obtained per sample time; and all biopsies were taken at sites distant from each other to guard against the possibility of biopsy-induced alterations in cell kinetics.

Samples from normal tissue were also obtained at each sample time, usually skin or lymphocytes, but sometimes it was possible to obtain samples of normal tissue immediately adjacent to the tumor such as normal mucosa (in the case of squamous carcinoma of the mouth) or vagina (in carcinoma of the cervix). These tissues were used to detect DAG-induced kinetics changes in normal tissues and to aid in determining the relative tumor cell DNA contents in the histograms. It was never possible to obtain the more toxicity-relevant normal tissue samples such as bone marrow or gastrointestinal epithelium; however, side effects in these studies were rare and mild.

DAG-induced S-Phase Kinetics Effects. A set of typical DNA FMF histograms can be seen in Chart 1. The data for the

![Chart 1](chart1.png)

Chart 1. DNA FMF histograms showing kinetic variability at different times after 100 mg DAG per sq m were administered to a patient with carcinoma of the anus (CA OF ANUS). CV, coefficient of variation.
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carcinoma of the anus have been normalized and computer drawn to indicate which portions of each histogram represent the \( G_1 \), \( S \), and \( G_2-M \) phases of the cell cycle. The untreated baseline biopsy sample contained 14.2% S-phase cells. Twenty-two hr after 100 mg DAG per sq m were administered to the patient, there were 17.6% S-phase cells. At 26 hr, the S-phase fraction had increased to 22.6% (or 160% more cells in S phase than at the baseline sample time). By plotting the percentage of S-phase cells versus time after DAG treatment, data can be presented as in Chart 2. This patient had 15% S-phase cells prior to receiving DAG. At 6 hr post-DAG treatment, the percentage of S-phase cells had decreased to 10% and then increased to a peak of 30% (2-fold increase) at 31 hr after DAG exposure.

A 1.9-fold increase in the S-phase fraction of tumor cells occurred at 30 hr post-DAG treatment in a patient with adenocarcinoma of the rectum (Chart 3A). There were 58% cells in S phase 30 hr post-DAG exposure compared to 30% in the baseline sample. A 1.3-fold increase was observed in the skin samples at the same hr. The patient whose data are presented in Chart 3B was treated with DAG and studied on 2 separate occasions, first in December 1979, which resulted in a 1.6-fold increase in the percentage of S-phase cells 26 hr post-DAG treatment, and again in June 1980, resulting in a 1.6-fold increase in S phase, but this time the peak occurred at 31 hr.

The DAG-induced kinetics changes in 2 patients with tumors of the head and neck are shown in Chart 4. In a patient with a metastatic carcinoma of the larynx (Chart 4A), the fraction of cells in S phase peaked at 24 hr post-DAG treatment at 43% (a 1.6-fold increase above the baseline of 27% S-phase cells). Two tumor biopsies were obtained, both at the 24- and 28-hr sample times, and each is plotted on the graph. No increase in the S-phase fraction was observed at 24 hr in the skin sample. A squamous carcinoma of the floor of the mouth (Chart 4B) exhibited a 1.5-fold increase in the S-phase fraction at 29 hr after DAG was administered. Although normal skin showed an increase in S phase at 24 hr, the fraction of cells in S phase had decreased to baseline levels by the time that the S phase peaked in the tumor.

The data from 3 patients with carcinoma of the cervix are shown in Chart 5. In the first patient (Chart 5A), a 2-fold increase in the S-phase fraction was observed at 36 hr post-

![Chart 2](image)

![Chart 3](image)

![Chart 4](image)

![Chart 5](image)
DAG exposure. The fraction of cells in S phase increased from 18 (pretreatment) to 36% at the peak hr. No change was detected in the kinetics of the normal vagina samples. The patient whose data are shown in Chart 5B was studied on 2 occasions. In September 1980, data from 2 pretreatment biopsies indicated an S-phase fraction of 14%. By 27 hr after DAG was administered, there were 33% S-phase cells (a 2.4-fold increase). When treated again in October 1980, a 1.9-fold increase in the S-phase fraction was observed at 24 hr. FMF samples of the normal vagina indicated no altered S-phase kinetics patterns. The data for the third patient (Chart 5C) was obtained over a longer sample time and indicated a 1.5-fold increase in S phase starting at 24 hr after DAG was given. The S-phase fraction remained elevated, reaching its highest value at 40 hr before falling. In this patient, there was also a 2-fold increase in the fraction of S-phase cells in the normal vagina samples at 40 hr.

In Chart 6 (carcinoma of the breast), it can be seen that there were 25% S-phase cells in the pretreatment baseline biopsy sample. The fractions in S phase decreased by 6 hr after DAG was administered and then began to increase, reaching a peak of 36% at 32 hr. Normal skin samples from this patient showed only slight fluctuations in cell kinetics parameters.

The results obtained on all 17 tumors were tabulated and can be seen in Table 1. For example, there were 3 patients with squamous carcinomas of the head and neck. The hr at which a peak in the S-phase fraction was observed ranged from 24 to 30 hr after DAG was given. In one patient (JD7), the fraction of cells in S phase went from 27% in the pretreatment baseline biopsy to 43% at the peak hr (Column 4). This represented a 1.6-fold enrichment or a 60% net increase in the fraction of cells in S phase (Columns 5 and 6). In general, the data for most other cancer types also indicated that the peak S-phase hr varied from patient to patient and that the net increase in the percentage of S-phase cells at the peak hr ranged from a low of 30% (patient CV13) to a high of 240% (DF16).

The kinetics in the melanoma was not altered at all by the DAG treatment; and in addition, the DNA FMF histograms from 2 patients with squamous carcinomas of the cervix (Al6 and GH18) could not be analyzed mathematically because the biopsy samples contained multiple clones of tumors, having widely different and overlapping DNA contents. An example from one patient is shown in Chart 7. The G1 peak channel of the first clone (Channel 23) had a normal DNA content (i.e., it peaked in the same channel as companion normal vagina and skin samples). The G1 peak of the second clone was in Channel 26 and that of clone 3 was in Channel 30, representing factors of 1.13 and 1.3 times more DNA, respectively, than normal. Therefore, 15 of 17 tumors could be analyzed for DAG-induced kinetics changes, and in 14 of the tumors, S-phase enrichment was demonstrated.

**DISCUSSION**

Since several types of tumors can grow on or near the surface of the body, this offers an excellent opportunity for the study of drug-induced cell kinetics changes in vivo and for the testing of tumor kinetics-directed treatment schedules. With the advent of FMF instrumentation as well as tumor cell dissociation and fluorescence staining techniques, such studies can be performed simply and rapidly. A knowledge of the perturbed tumor kinetics and degree of enrichment of cells into a particular cell cycle phase may make it possible to individualize the drug treatment schedules in such patients.

In the studies reported here, we used DAG to perturb the cell cycle kinetics of human tumors in vivo. The DNA histograms of 15 of the 17 tumors studied were evaluable for DAG-induced kinetics changes. (Two of the 17 tumors contained multiple clones of cells and could not be analyzed mathematically.) In all but one of the 15 evaluable tumors studied, the degree of S-phase enrichment was 1.3 to 3.4 times higher at some peak
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Table 1

Comparison of changes in S-phase kinetics following treatment with DAG among 6 different tumor types

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Patient</th>
<th>Peak hr</th>
<th>% of S at peak/% of S base line</th>
<th>Degree of S-phase enrichment</th>
<th>% of increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous carcinoma of the head and neck</td>
<td>JD7</td>
<td>24</td>
<td>43/27</td>
<td>1.6</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>OM8</td>
<td>29</td>
<td>37/24</td>
<td>1.5</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>CV13</td>
<td>30</td>
<td>45/36</td>
<td>1.3</td>
<td>30</td>
</tr>
<tr>
<td>Melanoma</td>
<td>S20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous carcinoma of the skin</td>
<td>AR1</td>
<td>35</td>
<td>44/29</td>
<td>1.5</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>AR2</td>
<td>36</td>
<td>37/27</td>
<td>1.4</td>
<td>40</td>
</tr>
<tr>
<td>Adenocarcinoma of the rectum</td>
<td>PB5</td>
<td>30</td>
<td>58/30</td>
<td>1.9</td>
<td>90</td>
</tr>
<tr>
<td>Squamous carcinoma of the anus</td>
<td>EF12</td>
<td>31</td>
<td>31/19</td>
<td>1.6</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>HS3</td>
<td>31</td>
<td>30/15</td>
<td>2.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>EF11</td>
<td>26</td>
<td>21/13</td>
<td>1.6</td>
<td>60</td>
</tr>
<tr>
<td>Squamous carcinoma of the cervix</td>
<td>RH19</td>
<td>40</td>
<td>34/21</td>
<td>1.6</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>EM14</td>
<td>27</td>
<td>33/14</td>
<td>2.4</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>A16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EM15</td>
<td>24</td>
<td>31/17</td>
<td>1.9</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>DF16</td>
<td>53</td>
<td>27/8</td>
<td>3.4</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>GH18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma of the breast</td>
<td>RS9</td>
<td>31</td>
<td>36/25</td>
<td>1.5</td>
<td>50</td>
</tr>
</tbody>
</table>

* Ratio from Column 4.
* No changes in kinetics values.
* Multiclonal tumors; histogram analysis not possible.

hr after DAG was administered. Presumably, an S-phase drug administered to these patients at the peak S-phase enrichment times would have 1.3 to 3.4 times greater effectiveness.

The hr at which the S-phase peak occurred was usually variable among patients with the same tumor type and among tumors of different types (Table 1). It is important to note that the S-phase peak hr also varied within some patients studied on 2 different occasions. For example, in a patient with squamous carcinoma of the anus (EF11), when first studied, the S-phase peak occurred at 26 hr after DAG was given. In a subsequent study in the same patient 6 months later (EF12), the S-phase peak occurred at 31 hr (Chart 3B; Table 1). In another patient with squamous carcinoma of the cervix, the S-phase peak occurred 27 hr after the first DAG treatment (Table 1, EM14) and at 24 hr after the second treatment (Table 1, EM15) which occurred only 6 weeks later. This is only a 3- to 5-hr difference; nevertheless, if either patient had been treated subsequently by the schedule directed by the first study, the S-phase peak may have been missed. This points out the value and importance of obtaining serial biopsies when using kinetics-directed treatment schedules.

The DAG-induced S-phase kinetics changes observed in the in vivo studies of human tumors are in excellent agreement with previously reported in vitro and in vivo studies (2, 12–14). It is not known whether the DAG treatment itself also induces any direct cell kill in these patients, and that must be studied. However, since we used doses which progressed up to 100 mg DAG per sq m before observing changes in kinetics and because the blockades were reversible, the data suggest the absence of (or minimal) cytotoxicity. Nevertheless, the point still must be tested.

Prior to the more universal availability of FMF instruments, others reported results of kinetics-directed treatment schedules in patients, usually assayed by radioactive isotope techniques (1, 4, 7, 9). We have used BLEO to induce partial synchrony at the S-G2 boundary (1). Tumors were biopsied and the fraction of cells in the S phase was determined at different times after BLEO treatment. In the melanoma patients studied, the fraction of cells in the S phase peaked at 2 to 3 days after BLEO and was between 1.5 and 3.7 times higher than in pretreatment tumor populations.

Lampkin et al. (9) have used a kinetics-directed treatment schedule and achieved enrichment or partial synchrony in a patient with acute lymphoblastic leukemia. Seventy-two hr after a single injection of ara-C, they observed an increase from 18 to 33% in the labeling index of leukemia lymphoblasts. At that time, vincristine was administered. After this schedule was repeated 3 times, the patient was in remission and was put on
that great variability in kinetics is normally found in tumors; this was followed 12 hr later by irradiation, and they have achieved greater than 90% response rates in tumors of the head and neck. Ernst and Killman (6) have reported the partial synchrony of human leukemic blast cells in vivo with methylprednisolone. The drug caused the reduced progression of tumor cells from G₂ phase to S phase. In addition, Gillette et al. (7), Mauro and Madoc-Jones (10), Rajewsky (15), and Dethlefsen (4, 5) have reported a rather high degree of synchronized S-phase cells (50 to 70% of the total population) after treatment of experimental animal systems in vivo with hydroxyurea.

In patient studies such as these, it is perhaps equally important to point out the problems which one encounters. For example, all of the patients used in our study had failed extensive prior therapy. Such patients are less willing to participate in studies requiring 5 to 7 biopsies of both normal and tumor tissue over a 2- to 4-day period. In addition, it is not always possible to obtain a statistically valuable number of patients with a particular tumor type, and that necessitates the study of whichever type becomes available. It should be pointed out, however, that in the present study, kinetics changes were induced by DAG in 5 different tumor types and this suggests that DAG may be useful in kinetics-directed drug combination treatments in a wide range of solid tumors.

Aside from the clinically related problems, many technical problems remain to be solved. The presence of multiple clones of cells in some tumor DNA histograms (Chart 7) makes it impossible to study kinetics changes in such patients. Cell survival assays such as reported by Salmon et al. (16) and Von Hoff et al. (20) to monitor the effectiveness of therapy are now available, but often one cannot obtain enough biopsy material for both the kinetics and cell survival assays. Another major problem is the degree of normal (unperturbed) variability of cell kinetics parameters at various sites within a tumor. From the work of Tannock (18) and Schiffer et al. (17), it is known that parameters such as growth fraction and fraction of cells in S phase may vary at different sites within a tumor for reasons of vascularity, oxygen content, and nutrition. Therefore, the kinetics values at different sites within a tumor could vary enough to cause one to erroneously conclude that a particular drug treatment had caused enrichment of cells into S phase.

Because of the bearing that this phenomenon may have on these kinetics-directed schedules, we are (in a separate study) comparing the kinetics parameters at different sites within a tumor. To date, 25 patients whose tumors ranged in length from 0.6 to 8 cm have been studied. An example of the results from one of the larger tumors is shown in Chart 8. The tumors were divided into sections of approximately 1 cm or less and in fact all normal tissues tested thus far, had smaller S-phase fractions than in the tumors. The processing and FMF analyses of multiple serial biopsies that we can detect such changes in the kinetic parameters. Although the 17 tumors in our kinetics-directed schedule studies were all under 5 cm, it is still possible that at least some of the degree of enrichment into S phase was due to site kinetics differences within the tumors and not entirely the result of DAG-induced kinetics changes. Therefore, the data suggest that a limit might be placed on an observed degree of enrichment before it can be stated with confidence that it represents a real drug-induced change in kinetics.

CONCLUSION

It is important to point out that the goal at this time was not to completely synchronize the tumor cell population. Instead, it was: (a) to use relatively noncytotoxic doses of DAG to perturb the cell kinetics patterns of human tumor cells in vivo; (b) to determine the degrees of enrichment of cells into S phase; and (c) to determine the times at which the enrichment occurred. We have good evidence in 14 of 17 patients studied that we can detect such changes in the kinetic parameters. The processing and FMF analyses of multiple serial biopsies from these patients was rapid, taking less than an hr, and should allow the administration of an appropriate S-phase agent at a time when it would be more effective. The significance of the study of such kinetics-directed schedules, therefore, is that the patient’s own tumor cell kinetics might be used to derive an optimal drug treatment regimen for his own tumor, thus individualizing his therapy.
That is not to say that such treatment schedules should or could be universally used now. Enough technical problems exist to keep this form of therapy in the experimental stages for some time to come. Nevertheless, by performing the studies at this time, we are able to identify the associated problems and questions and to work on solutions for them. As progress is made in this area, we should eventually be able to test the effectiveness of kinetics-directed treatment schedules and compare the responses to other more conventional treatment regimens.

ACKNOWLEDGMENTS

The authors wish to thank David Egle and Ken Shilkun for their excellent technical assistance and R. Kenworthy for assistance in preparation of the manuscript.

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