The Effects of Cyclophosphamide, Ketoconazole, Aclacinomycin-A, Methotrexate, and Scheduled Methotrexate–5-Fluorouracil Combination Chemotherapy on the Transplantable R-3327 Prostatic Adenocarcinoma in the F_1 Hybrid Male Rat

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Male F_1 hybrid rats bearing the R-3327 transplantable prostatic adenocarcinoma demonstrating similar growth patterns within the original sample of animals were carefully separated into control and treatment groups. This assured treatment of tumors with similar cell kinetics within each group. In the first study, two separate drug protocols were investigated by intraperitoneal injection, namely cyclophosphamide (100 mg/kg) once every 4 weeks for 8 weeks and scheduled methotrexate (7.5 mg/kg) followed in 90 minutes by 5-fluorouracil (50 mg/kg) once each week for 8 weeks. Excellent suppression of tumor growth was obtained with each treatment protocol. Both were significant at the 0.01 level. In the second study, methotrexate (100 mg/kg) intraperitoneally once each week for 6 weeks, aclacinomycin-A intraperitoneally once each week for 4 weeks, and ketoconazole (60 mg/kg) via gavage 5 times a week for 6 weeks were administered to the animals in each respective group. Aclacinomycin-A and ketoconazole showed significant suppression of tumor growth at the 0.01 and 0.05 levels, respectively. Methotrexate suppressed tumor growth, but did not reach levels of significance over the duration of the study ($0.2 < P < 0.3$).

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IT IS WELL KNOWN that the Dunning R-3327 transplantable rat prostatic adenocarcinoma serves as an excellent animal model for the study of prostatic cancer as the histology, histochemistry and hormone sensitivity parallel the human tumor very closely.[1,2] In this regard, many treatment modalities have been directed against this tumor.[3–10] Yagoda reviewed the clinical response to nonhormonal cytotoxic agents in the treatment of prostatic adenocarcinoma of the human,[11] and although the response is not as dramatic, these agents are basically the drugs that demonstrate tumor suppression in the rat model.

In a previous report, we investigated the Dunning transplantable tumor response to cyclophosphamide and to scheduled methotrexate–5-fluorouracil (5-FU) and found these agents to be very effective at suppressing tumor growth.[12] The theoretical basis for scheduling methotrexate and 5-FU was advanced by Cadman *et al.*[13] and relates to the cellular accumulation of ribonucleotide derivatives of 5-FU, then incorporation into RNA in preparations previously treated with methotrexate.[13]

The work presented herein is a continuation and extension of the previous study but with two new drug protocols. In addition to investigating methotrexate alone, ketoconazole, and aclacinomycin-A were also studied.

Materials and Methods

Selection of Animals

The animals studied were male F_1 hybrid rats (Copenhagen male and Fisher female) bearing the hormone sensitive slow growing transplantable Dunning R-3327 adenocarcinoma injected subcutaneously into their flanks. These animals were obtained from the Papanicolaou Cancer Research Institute, Miami, Florida. Even

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though the animals were implanted at the same time, the tumors will not all grow at the same rate. To insure treating tumors of basically similar cell kinetics, we separated the animals into groups with tumors growing roughly at similar rates. The method for achieving this was outlined in our previous work,[12] and basically involves housing and observing the animals for some time, due to the fact that when the animals are received from the Papanicolaou Institute, the tumors are in most instances impalpable. After the tumors reach reasonable sizes, tumor dimensions are measured with a Vernier caliper and the animals are then separated into groups of similar tumor sizes. This insures that apparent treatment effect is not due to variations in cell kinetics between the treatment and control groups.

Selection of Drug Dosages

The final drug schedules and dosages were arrived at by using test animals and noting the effects produced on the animals' ability of continued growth by recording weight, the drugs' ability to suppress cell growth and division by recording white blood cell counts, and finally the overall effect upon the animals by noting survival. Several test animals were used as above to determine the safe dosages and schedules for methotrexate, 5-FU, cyclophosphamide, and aclacinomycin-A. The final dosage for ketoconazole was selected by doubling a previous dosage (30 mg/kg) that had failed to produce a depression of the blood testosterone level. It should also be emphasized that an adequate control group should always be used with each experiment, as even some of these drug free control animals will occasionally die for no apparent reason.

The final dosage schedule for cyclophosphamide of 100 mg/kg intraperitoneally (IP) once every 3 to 4 weeks is not different from the literature.[7,8] Our 5-FU dosage of 50 mg/kg IP once each week is in a cumulative manner similar to the literature[7,8] values of 120–180 mg/kg once every 3 weeks. However, Block *et al.* after noting that 17 of the 19 animals died at a dosage of 2 mg/kg methotrexate every 3 weeks, finally settled on 1.3 mg/kg methotrexate every 3 weeks for their animals.[7,8] This is very different from our doese of 7.5 mg/kg IP once each week, and our animals did very well in both the previous study[12] and the current work, once again emphasizing the importance of an adequate control group.

Therapeutic Groups

Aclacinomycin-A: Seven animals were entered into this treatment group. Each animal received aclacinomycin-A, 8 mg/kg IP, each week for 4 weeks. Five additional animals were assigned to the control group and they received 1 ml normal saline IP each week for 4 weeks. Since the effects of aclacinomycin last for some time, these animals were not killed until 2 weeks after the last dose of the drug. This gave an overall treatment time of 6 weeks.

Ketoconazole: Seven animals were assigned to this treatment group and each animal received ketoconazole, 60 mg/kg via gavage, 5 times each week for 6 weeks. The control group was the same as the aclacinomycin-A control group above, since all these animals (including aclacinomycin-A and ketoconazole) were taken from the same group with similar growth patterns.

Methotrexate: Seven animals were assigned to this treatment group and each animal received methotrexate, 7.5 mg/kg IP, each week for 6 weeks. Seven animals were entered into this control group and they received 1 ml normal saline IP each week for 6 weeks. These 14 animals had different growth rates than the aclacinomycin-A and ketoconazole animals above, so these groups cannot be compared.

After the treatment protocols were completed, the animals were weighed, killed, the tumors dissected out and the weights recorded. In addition, serum testosterone levels were measured on some control and ketoconazole animals.

Results

The experimental results of the present study and the previous study are shown in Tables 1, 2, and 3. After the animals had been selected for the various treatment groups, as indicated under Materials and Methods, some means of ascertaining the initial tumor mass had to be found. This was done by measuring the three perpendicular axes of each tumor and the mass was calculated according to the formula

$$M = \frac{\pi}{6} \delta abc$$

where δ = 1.05 gm/ml is taken as the density of the tissue and a, b, c represent the respective three perpendicular diameters.

The statistic used in the calculation of significance was the fractional increase of each tumor over the duration of the treatment protocol. That is, the quantity

$$F = \frac{Mf - Mi}{Mi}$$

where F = fractional increase of tumor, Mi = initial calculated mass of tumor, Mf = final recorded mass of tumor, was calculated for each tumor.

Thus, an F value could be generated for each of the control animals as well as for each of the treatment animals. The two-tail *t*-test was then used to calculate

TABLE 1. Aclacinomycin-A and Ketoconazole

Treatment group	Mean initial calculated tumor mass*	Mean final measured tumor mass	Mean F value*	Significance (*P*)†
Control‡	1.41 ± 0.67	22.40 ± 4.30	22.65 ± 3.88	
Aclacinomycin-A	4.26 ± 0.58	22.44 ± 5.97	3.75 ± 0.88	$P < 0.01$
Ketoconazole	3.18 ± 1.23	22.67 ± 8.03	7.74 ± 1.54	$P < 0.05$

* See text under Results.
† Significance between control and treatment F values, see text under Results.
‡ All means ± SE.

significant differences between the control set of F values and the treatment set of F values for the various treatment regimes. This method allowed us to eliminate the actual mass of the tumors from consideration since, even though the animals were separated into groups with similar tumor sizes, there still will be some variations. Through the F values, the more important statistic of fractional or percentage increase of each tumor is assessed irrespective of the exact starting mass which might be somewhat different for each animal and/or treatment groups.

As can be seen from Table 1, aclacinomycin-A and ketoconazole were both very effective treatments in suppressing tumor growth, aclacinomycin-A being signficant at the 0.01 level and ketoconazole at the 0.05 level. Five animals were entered into the control group and all animals survived. Seven animals were entered into the aclacinomycin-A group and five usable animals survived, one died, and one had the tumor partially destroyed by its cage mates. Seven animals were entered into the ketoconazole group and three usable animals survived, two died, and two had their tumors partially destroyed by their cage mates. Most of the animals looked well and most gained weight. We recognize the significance of the small number of usable animals in the treatment group. We believe that the results are valid, however, since we carefully selected animals with roughly the same growth rate for both the treatment and control groups. In addition, as stated above, by using the statistic F, we have observed the fractional increase of each tumor, irrespective of starting mass. These two methods essentially eliminate the random nature of the selection process. The blood testosterone level in the ketoconazole rats receiving 60 mg/kg was not different from the control rats. However, it was depressed to very low levels in rats that had undergone bilateral orchiectomy.

Table 2 shows that methotrexate given IP at 7.5 mg/kg once each week for 6 weeks did suppress tumor growth but not to levels of significance ($0.2 < P < 0.3$) over the duration of the study. Seven animals were entered into the methotrexate treatment group and five usable animals survived, one died, and one had the tumor partially destroyed by its cage mate. Seven animals were assigned to the methotrexate control group and six usable animals survived, one animal died. Most of the surviving animals gained weight and looked well.

Table 3 shows the work from the previous study[12] in which we found excellent tumor suppression with both cyclophosphamide and scheduled methotrexate–5-FU, both being significant at the $P < 0.01$ level. Of the 11 animals assigned the cyclophosphamide treatment group, 3 of the animals died. Curiously, five of the nine animals assigned to the cyclophosphamide control group died. The surviving animals were not different in initial mean tumor sizes and initial body weight from the original group of nine.[12] The methotrexate–5-FU treatment group had nine animals assigned and only three died. Of the six animals assigned to the methotrexate–5-FU control group, only one died. In all of these groups, most of the surviving animals gained weight, looked well and, in addition, had normal complete blood counts and platelet counts.[12]

Discussion

This work indicates that excellent suppression of tumor growth in the transplantable Dunning R-3327 rat prostatic adenocarcinoma can be obtained with cyclo-

TABLE 2. Methotrexate

Treatment group	Mean initial calculated tumor mass*	Mean final measured tumor mass	Mean F value*	Significance (*P*)†
Control‡	1.74 ± 0.59	6.33 ± 2.03	4.46 ± 0.89	
Methotrexate	4.91 ± 1.67	14.0 ± 5.35	2.24 ± 0.54	$0.2 < P < 0.3$

* See text under Results.
† Significance between control and treatment F values, see text under Results.
‡ All means ± SE.

TABLE 3. Cyclophosphamide and Scheduled Methotrexate–5-FU

Treatment group	Mean initial calculated tumor mass (gm)*	Mean final measured tumor mass (gm)	Mean F value*	Significance (*P*)†
Control‡	6.47 ± 1.37	56.75 ± 5.42	9.51 ± 2.05	
Cyclophosphamide	5.77 ± 1.97	17.18 ± 5.50	2.35 ± 0.57	$P < 0.01$
Control	0.90 ± 0.26	28.02 ± 7.63	40.09 ± 9.26	
Scheduled methotrexate–5-FU	1.19 ± 0.21	7.85 ± 1.23	7.78 ± 2.68	$P < 0.01$

* See text under Results.
† Significance between control and treatment F values, see text under Results.
‡ All means ± S.E.

phosphamide (100 mg/kg IP once every 4 weeks), ketoconazole (60 mg/kg via gavage 5 times a week for 6 weeks), aclacinomycin-A (8 mg/kg IP once each week for 4 weeks), and scheduled methotrexate–5-FU (7.5 mg/kg and 50 mg/kg, respectively, IP once each week for 8 weeks). Methotrexate given alone (7.5 mg/kg once each week for 6 weeks) suppressed tumor growth, but did not reach levels of significance over the duration of the study.

The rationale for scheduling methotrexate–5-FU (*i.e.*, giving methotrexate followed in 90 minutes by 5-FU) is based on a synergistic response when this scheme is used. The theoretical basis was advanced by Cadman *et al.*,[13] who proposed that methotrexate increased the cellular concentration of phosphoribosylpyrophosphate in cells that use this agent as a ribose and phosphate donor. The increased concentration of this agent leads to increased cellular accumulation of 5-FU ribonucleotides when 5-FU is given at a delayed time. Cell killing as apparently related to the increased incorporation of the 5-FU triphosphate nucleotides into RNA.[13]

Ketoconazole, an antifungal agent, was studied as a possible antiprostatic adenocarcinoma agent since it is known to cause depression of the blood testosterone level. Even considering the small number of animals, it appears to be an effective agent in the rat in the dosages used. Since we were not able to obtain depression of the blood testosterone levels, it is interesting to speculate whether the ketoconazole has a direct effect on the tumor. However, this probably represents a time dependent effect, and since ketoconazole has other endocrine effects the lack of suppression of the testosterone level provides only very weak evidence of direct cytotoxicity of ketoconazole.

Aclacinomycin-A is an anthracycline which inhibits DNA, RNA, and protein synthesis. It seems to have excellent suppression of prostatic tumor growth in the rat.

We mentioned earlier in this article in the Results section, that although the animals in each individual study group demonstrated similar growth patterns in most cases, one study group cannot be compared to another study group because the growth patterns among groups are different. Therefore it should be specifically stated that this study has demonstrated the effectiveness of certain drug treatments but has not proven the superiority of any one of the treatments, be it hormonal or chemotherapeutic.

Clinical trials should be estimated to determine safe dosages and the effectiveness of these agents in the treatment of disseminated human prostatic adenocarcinoma.

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