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Chemoprevention of spontaneous tumorigenesis in nullizygous p53-deficient mice by dehydroepiandrosterone and its analog 16α-fluoro-5-androsten-17-one

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Transgenic mice with both alleles of the *p53* tumor suppressor gene product 'knocked out' by gene targeting are susceptible to early development of tumors, chiefly lymphomas and sarcomas. Compared with the control group, administration of dehydroepiandrosterone (DHEA) at 0.3% of the diet to male p53-deficient mice extended their lifespan by delaying death due to neoplasms (from 105 to 166 days on study, $P = 0.002$), primarily by suppressing lymphoblastic lymphoma (from 45 to 6% of neoplastic deaths, $P = 0.010$). Treatment with a synthetic DHEA analog, 16α-fluoro-5-androsten-17-one (compound 8354), at 0.15% of the diet also increased lifespan, to 140 days for mice that developed tumors ($P = 0.037$). The effects of these steroids on lifespan and tumor development did not appear to be strongly related to inhibition of food consumption and weight gain, in that a group pair-fed with control diet to the reduced food consumption of the DHEA-treated group developed and died of the same types of neoplasms at the same rate as the controls fed *ad libitum*. The chemopreventive effect of these steroids has been proposed to be due to suppression of DNA synthesis by inhibition of glucose 6-phosphate dehydrogenase, the rate-limiting enzyme of the pentose phosphate pathway. Although DHEA and its analog are strong non-competitive inhibitors of this enzyme *in vitro*, treatment with DHEA did not deplete cellular nucleotide pools in the liver, as would have been predicted. The chemopreventive effect of DHEA in this model may be due to steroid-induced thymic atrophy and suppression of T cell lymphoma, permitting these mice to survive long enough to develop tumors with longer latency.

Introduction

Dehydroepiandrosterone (DHEA*) is an adrenocortical steroid of unknown physiological function. As its sulfate derivative it is by far the most abundant steroid in humans, with circulating levels in young adults of the order of 5–7 mM; these levels inexorably decline with age to 5% of peak values by the ninth decade (1). Reports have suggested an inverse relationship between DHEA levels, unrelated to age, and the risk for various cancers (2). Exogenously administered DHEA has been shown to have broad spectrum cancer preventive activity in a number of spontaneous and chemically induced rodent carcinogenesis models (3). The potential clinical utility of DHEA is limited by its role as a precursor for the synthesis of testosterone and estrogens. However, 16α-fluoro-5-androsten-17-one (compound 8354) (4), a synthetic DHEA analog that cannot be metabolized to androgens and lacks the liver toxicity and many of the hormone-related side effects of DHEA, is scheduled for phase I pharmacokinetic studies (5). Compound 8354 shares with DHEA its chemopreventive activity in cancer models and is an even more potent non-competitive inhibitor *in vitro* of glucose 6-phosphate dehydrogenase (G6PDH), the rate-limiting enzyme in the pentose phosphate pathway; modulation of this enzyme activity has been hypothesized to play a critical role in the chemopreventive activity of these steroids (3).

Transgenic mice in which the function of the p53 tumor suppressor has been 'knocked out' by gene targeting inevitably develop neoplasms (chiefly lymphomas) by 10 months of age (6). We have previously shown that dietary intervention can delay tumor development and lengthen lifespan in these mice, despite their genetic liability (7,8). The present study was designed to confirm and extend our finding that DHEA is a highly effective chemopreventive agent in this model (8) and to investigate its mechanism of action. To this end we administered DHEA in the diet to nullizygous p53-deficient ($p53^{-/-}$) mice and compared its effects on spontaneous tumorigenesis with those of the DHEA analog compound 8354. To control for the inhibitory effects of DHEA on food consumption (and hence weight gain), another group of mice (designated 'pair-fed to DHEA') was fed control diet and restricted to the average amount of food consumed daily by the mice in the DHEA group. Both steroids significantly delayed spontaneous tumor development (especially lymphoblastic lymphoma) in these genetically susceptible mice, suggesting that the chemopreventive activity of these compounds, at least toward lymphoma, does not require the *p53* tumor suppressor gene product.

Materials and methods

Animals and diets

Seventy-eight male $p53^{-/-}$ mice (GenPharm International, Mountain View, CA) were individually housed in polycarbonate cages on hardwood bedding and maintained on a 12 h light/dark cycle at 24°C. The colony from which these mice were obtained was generated from 129/Sv–, C57BL/6 chimeric founders (6) with back crosses to C57BL/6 mice for four generations. Following a 2-week acclimation period after receipt, during which time all animals were fed AIN-76A semi-purified diet *ad libitum*, the mice (now ~10 weeks of age) were randomized to one of four dietary treatment groups (18–20 mice/group): (a) control, which received AIN-76A diet with no added agents; (b) DHEA, which received AIN-76A diet containing 0.3% (w/w)

***Abbreviations:** DHEA, dehydroepiandrosterone; compound 8354, 16α-fluoro-5-androsten-17-one; G6PDH, glucose 6-phosphate dehydrogenase; $p53^{-/-}$, nullizygous p53-deficient; $p53^{+/+}$, wild-type p53.

DHEA; (c) 8354, which received AIN-76A diet containing 0.15% (w/w) compound 8354; (d) pair-fed to DHEA, in which each mouse received the average amount of control AIN-76A diet as consumed daily by the mice in the DHEA group during the previous week. This last group was designed to control for the reduced consumption of food by the steroid-treated mice. All diets were purchased from Research Diets, Inc. (New Brunswick, NJ). This formulation of AIN-76A consisted of 50% sucrose (w/w), 20% casein, 10% corn starch, 5% Lodex 10, 5% corn oil, 5% cellulose, 0.3% *dl*-methionine, 0.2% choline bitartrate and AIN-76A vitamin and salt mixes. It was manufactured as 1-g pellets after addition of 0.5% each of silicon dioxide and magnesium stearate to facilitate tableting. DHEA was purchased from Sigma Chemical Co. (St Louis, MO). Compound 8354 was a generous gift of Dr Arthur G.Schwartz. (Temple University, Philadelphia, PA). Control diet or diets mixed with DHEA or compound 8354 were tableted in 10-kg lots, stored at room temperature and (except for the group pair-fed to DHEA food consumption) administered *ad libitum* in standard feeders in weekly aliquots; weekly food consumption by an individual mouse was estimated from the weight of food remaining in the feeder plus that of any pieces of pellet that had fallen into the bedding. All mice also received distilled water *ad libitum*. Food intake and body weight were recorded weekly.

Spontaneous tumorigenesis study

All mice were observed daily for clinical signs of ill health. Moribund mice were killed by CO_2 asphyxiation. All animals that were killed or found dead were necropsied and their tissues were fixed in neutral-buffered 10% formalin, embedded in paraffin and sectioned at 4–6 μm. Sections stained with hematoxylin and eosin were histopathologically analyzed to determine cause of death and tumor type. Numbers reported for individual tissues were sometimes less than the group size because of losses during tissue processing. The survival distributions for the four treatment groups were compared using the Cox proportional hazards model (9); computations were performed using the computer program developed and described by Thomas *et al.* (10). Tumor multiplicities were compared using the non-parametric Kruskal–Wallis and Wilcoxon rank-sum procedures (11). Comparisons among the four proportions of tumor incidence or tumor cause of death were made using the χ^2 test; pairwise comparisons were made using the Fisher exact test (12).

Quantification of nucleotide pools

$p53^{-/-}$ mice and wild-type p53 ($p53^{+/+}$) littermates were fed either control diet or diet containing 0.3% DHEA for 4 weeks before the mice were killed with CO_2 and the liver quickly frozen in liquid nitrogen and stored at –80°C. The tissues were extracted with trichloroacetic acid, and nucleotides and deoxynucleotides were separated by reversed phase high performance liquid chromatography coupled with diode array detection as described (13). Treatment group effects on nucleotide levels were compared by unpaired *t*-tests and one-way analysis of variance.

Results

Consistent with the known anti-obesity activity of these steroids (14), administration in the diet of either DHEA or the related steroid compound 8354 significantly reduced average weight gain in treated $p53^{-/-}$ mice compared with mice fed control diet (Figure 1). Much but not all of this inhibition of weight gain appeared to be due to decreased food intake, with average weekly consumption by the mice in the steroid-treated groups rising after the first 2 weeks on study to ~64% of that of the control group during weeks 3–12. Mice that were pair-fed to the food consumption of the DHEA group also weighed significantly less than the control group, but slightly more than the steroid-fed mice. After the first 2 weeks on study, food consumption/100 g body wt was similar among the treatment groups for 5 weeks and then actually higher in the steroid-fed mice. These observations conform with the reduced efficiency of food utilization that has been reported in DHEA-treated animals (14).

Administration of steroids in the diet to $p53^{-/-}$ mice resulted in significant differences in the Kaplan–Meier survival curves for the respective treatment groups (Figure 2). The majority of mice, regardless of treatment, spontaneously developed neoplasms and died from them, with the exception of the following: one DHEA-treated mouse, which died of hydronephrosis; two mice treated with compound 8354, which died of infection/septicemia; one pair-fed mouse and two 8354 mice, for which the cause of death could not be determined. As previously reported (8), administration of DHEA at 0.3% of the diet to $p53^{-/-}$ mice significantly delayed tumor-related mortality relative to controls ($P = 0.002$). The mean time on study for the DHEA-treated mice that died of neoplasms was 166 days, compared with 105 days for the control mice (Table I). Compound 8354 given at an intermediate dose (0.15% of the diet) had a less significant effect relative to controls on tumor-related mortality ($P = 0.037$) and mean time to death

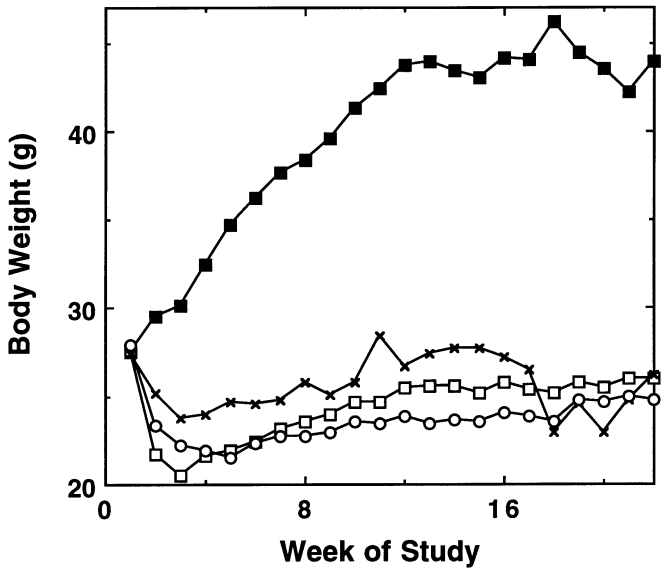

Fig. 1. Mean body weights by week of study of $p53^{-/-}$ mice fed control diet (closed squares), 0.3% DHEA (open squares) or 0.15% compound 8354 (open circles) or pair-fed to the food consumption of the DHEA group (crosses). $n = 18$–20 per group.

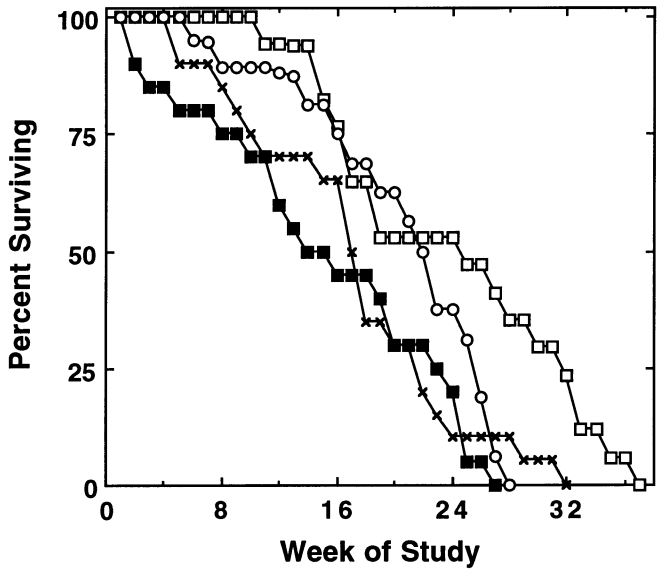

Fig. 2. Kaplan–Meier survival curves for $p53^{-/-}$ mice fed control diet (closed squares), 0.3% DHEA (open squares) or 0.15% compound 8354 (open circles) or pair-fed to the food consumption of the DHEA group (crosses) through 37 weeks of study. Both DHEA-treated ($P = 0.002$) and 8354-treated ($P = 0.037$) mice showed a statistically significant delay in mortality due to spontaneous tumor development. Mice pair-fed to the DHEA group did not live significantly longer than the control group ($P = 0.74$).

Table I. Effect of chemopreventive steroids on cause of death in p53-deficient mice

	Treatment group			
	Control	Pair-fed	DHEA	8354
Cause of death				
Neoplasm	20/20	19/20	17/18	16/20
Infection/septicemia	0	0	0	2/20
Other/undetermined	0	1/20	1/18	2/20
Mean days on study				
Mice with neoplasms	105	115	166	140
All mice	105	116	161	127
Cause of death (% neoplasm deaths)				
Hematopoietic neoplasm	15/20 (75)	15/19 (79)	8/17 (47)	11/16 (69)
Lymphoma				
Lymphoblastic	9/20 (45)	10/19 (53)	1/17 (6)[e]	4/16 (25)
Non-lymphoblastic[a]	5/20 (25)	3/19 (16)	5/17 (29)	5/16 (31)
Other hematopoietic neoplasm[b]	1/20 (5)	2/19 (11)	2/17 (12)	2/16 (12)
Sarcoma[c]	3/20(15)	2/19 (11)	8/17 (47)[f]	3/16 (19)
Other neoplasm[d]	2/20 (10)	2/19 (11)	1/17 (6)	2/16 (12)

[a]Mixed morphology lymphoma; atypical lymphoblastic lymphoma; follicular center cell lymphoma; blastic/stem cell lymphoma.
[b]Histiocytic sarcoma; myeloblastic leukemia; unclassified hematopoietic neoplasm.
[c] Hemangiosarcoma; myxosarcoma; sarcoma, not otherwise specified.
[d]Glial tumor; salivary gland myoepithelioma; thyroid tumor; testicular tumor; squamous cell carcinoma; liver tumor.
[e]Different from control and pair-fed, $P \leq 0.010$.
[f]Different from pair-fed, $P = 0.025$.

(140 days). The ability of DHEA and compound 8354 to delay tumor development and to increase survival time did not appear to be strongly related to the decreases in food consumption and weight gain caused by these steroids, given that the group pair-fed to the DHEA group died of tumors as rapidly as the control group (Figure 2), with a mean time to death of 115 days. Furthermore, mice fed compound 8354 weighed slightly less on average than the DHEA-treated mice for much of the study (Figure 1), but (as noted above) at the doses given compound 8354 was not as effective as DHEA in delaying neoplastic death (Figure 2).

As in previous studies (7,8), the majority of $p53^{-/-}$ mice fed the control diet died of hematopoietic neoplasms, with lymphoblastic lymphoma (based on morphology) the most common cause of death (Table I). The group pair-fed with control diet to the reduced food consumption of the DHEA group died from the same types of neoplasms as the control group. As shown previously (8), DHEA at 0.3% of the diet significantly inhibited the development of lymphoblastic lymphoma ($P = 0.010$), with only 1/17 tumor deaths in the DHEA group due to this type of lymphoma, compared with 9/20 tumor deaths in the control group (Figure 3 and Table I). Although fewer mice treated with compound 8354 died from lymphoblastic lymphoma (4/16 deaths from tumor) compared with the control group, this effect was not statistically significant at the dose given (0.15% of the diet) and with these group sizes. In this study DHEA appeared to have little effect on death due to other types of hematopoietic neoplasms but increased the proportion of mice that died from sarcoma. In particular, five DHEA-treated mice died from hemangiosarcoma, compared with one each in the control and pair-fed groups and two in the 8354 group; this increase in deaths from hemangiosarcoma with DHEA administration was seen in a previous study as well (8).

In addition to the inhibitory effects on lymphoblastic lymphoma development in $p53^{-/-}$ mice, steroid treatment resulted in a decrease in the number of mice that developed liver neoplasms (benign and malignant), from 8/20 and 6/20 in the control and pair-fed groups respectively to 2/18 and 1/20 in the DHEA and 8354 groups. In contrast, testicular neoplasms (2/17 in the DHEA group and 3/19 in the 8354 group) and prostatic sarcoma (1/18 in the DHEA group) appeared only in mice treated with steroids.

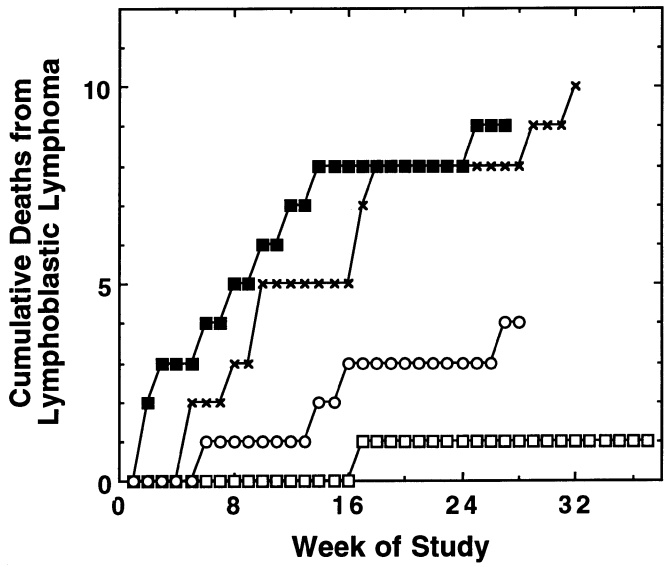

Fig. 3. Cumulative deaths from lymphoblastic lymphoma in $p53^{-/-}$ mice fed control diet (closed squares), 0.3% DHEA (open squares) or 0.15% compound 8354 (open circles) or pair-fed to the food consumption of the DHEA group (crosses). $n = 20$ per group, except $n = 18$ for DHEA. Significantly fewer deaths from lymphoblastic lymphoma were seen in mice fed DHEA ($P = 0.010$).

In this study neither DHEA nor compound 8354 had any effect on the proportion of mice that developed multiple primary neoplasms (benign or malignant) before they died, compared with controls (Table II). All mice that had at least one tumor died because a tumor and steroid treatment had no statistically significant effect on the proportion of mice that died from causes other than tumor. There was a trend for

Table II. Effect of chemopreventive steroids on numbers and types of neoplasms in p53-deficient mice

	Treatment group			
	Control	Pair-fed	DHEA	8354
No. of mice/group	20	20	18	20
Mice with neoplasms	20	19	17	16
Mice with multiple neoplasms	9	7	8	8
No. of neoplasms/group				
Total primary neoplasms	33	28	27	27
Total benign	3	4	3	4
Total malignant	30	24	24	23
Total malignant with metastasis	20	18	15	12

Table III. Effect of dietary DHEA on liver nucleotide pools in wild-type and p53-deficient mice

Nucleotide	Wild-type		p53-deficient	
	Control	DHEA	Control	DHEA
NAD	1.1 ± 0.7	3.5 ± 1.7[a]	3.4 ± 1.2[a]	2.7 ± 1.5
ATP	1.4 ± 0.2	3.8 ± 2.1	3.4 ± 1.3[a]	2.8 ± 1.0
dATP	1.1 ± 0.4	1.1 ± 0.4	0.8 ± 0.4	1.1 ± 0.3
dCTP	3.5 ± 0.8	2.8 ± 0.9	3.0 ± 1.1	2.5 ± 0.4
dTTP	4.9 ± 1.7	3.1 ± 1.2	3.9 ± 1.5	3.6 ± 1.8
dUTP	3.3 ± 0.9	3.7 ± 1.4	2.6 ± 1.1	1.9 ± 0.3[b]
dCMP	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.2	0.5 ± 0.2
dTMP	2.5 ± 0.4	1.8 ± 0.5	1.7 ± 0.5	1.8 ± 0.8

Values are mean ± SD, nmol/mg protein; $n = 3$–5 individual mice.
[a]Different from respective wild-type control group by unpaired *t*-test.
[b]Different from wild-type DHEA group by unpaired *t*-test.
There were no significant differences among treatment groups by one-way analysis of variance.

steroid treatment to decrease the proportion of malignant tumors that metastasized, but this effect was not quite statistically significant ($P = 0.065$ for the 8354 group compared with controls).

In addition to its effects on food consumption, body weight, survival time and tumor type, DHEA (but not compound 8354) administration caused adrenal cortical hyperplasia (four out of 15 tissues available for examination) or adrenal cortical atrophy (3/15) in some mice. Thymic cortical atrophy was also more common in the DHEA-treated animals (6/10) than other groups and reduced organ size may explain why only 10 tissues were available for histopathological examination for this group. Steroid treatment increased the incidence of endosteal hyperostosis in the skull and femur (11/18 and 16/18 respectively for DHEA and 14/20 and 19/20 for 8354 groups) compared with the control (0/19 and 2/20) and pair-fed groups (0/20 and 0/19). There was also a marginal increase in the incidence of granulocytic hyperplasia in the marrow in the DHEA (7/18) and 8354 (6/20) groups compared with the control (4/19) and pair-fed (2/18) groups.

The median time on study for all mice was 18 weeks. Of the 24 mice that died of lymphoblastic lymphoma, 79% died within the first 18 weeks (Figure 3). In contrast, 83% of the 18 mice that died of other types of lymphoma did so after the first 18 weeks. Except for the nine cases of hemangiosarcoma, of which only 44% survived past the first 18 weeks on study, other neoplasms also tended to cause death later than the median (62%). These results suggest that steroid treatment, by specifically suppressing the development of lymphoblastic lymphoma, permitted these mice to survive long enough to develop types of tumors with a later average time to morbidity.

DHEA and compound 8354 have been shown to be strong non-competitive inhibitors of G6PDH activity *in vitro* (4). Inhibition of G6PDH activity would be expected to result in depletion of cellular nucleotide pools (3). In a separate, short-term study, administration of DHEA in the diet at 0.3% for 4 weeks had no statistically significant effect in either $p53^{-/-}$ mice or their $p53^{+/+}$ littermates on NAD, ATP or deoxynucleotide levels in the liver by one-way analysis of variance (Table III). However, p53 status did appear to affect control NAD and ATP levels, which were higher in livers from p53-deficient mice compared with their wild-type littermates (Table III). By themselves, these data do not support the hypothesis that the chemopreventive effect of DHEA on spontaneous tumorigenesis in p53-deficient mice is a consequence of depletion of cellular nucleotide pools brought about by inhibition of G6PDH activity, at least with a 4-week treatment at the dose given.

Discussion

As shown previously (8), administration of DHEA at 0.3% of the diet significantly delayed tumor development and thereby extended lifespan in $p53^{-/-}$ mice (Figure 2). Compound 8354 at 0.15% of the diet had a lesser but still significant effect on survival. Consistent with previous reports of the effects of these compounds on food consumption and energy metabolism (15), DHEA and compound 8354 also significantly inhibited weight gain (Figure 1). Mice that were pair-fed to the DHEA group (to control for the reduced food intake seen in animals treated with DHEA) developed tumors and died as rapidly as the control group. Although we previously showed that enforced calorie restriction to 60% of *ad libitum* consumption (with

all diet components isonutrient except carbohydrates) also significantly inhibits weight gain and delays tumor development in this model (7), in the present study the reduced intake of all nutrients did not have the same inhibitory effect on spontaneous tumorigenesis. Thus, the decreases in food intake and weight gain that accompanied steroid administration do not seem to account for the chemopreventive effects of DHEA and compound 8354 on tumorigenesis in $p53^{-/-}$ mice.

Abundant evidence suggests that much of the chemopreventive activity of DHEA and its synthetic analogs may be related to inhibition of G6PDH activity (3). Among the consequences of inhibiting this enzyme, and hence flux through the pentose phosphate pathway, would be depletion of ribose 5-phosphate and NADPH (which are used in the synthesis of ribo- and deoxyribonucleotides) and therefore retardation of cell cycle progression due to inhibition of DNA synthesis (3). DHEA has been shown to act *in vitro* as a non-competitive inhibitor of G6PDH with a K_i of 18.7 μM (4). Its synthetic analog compound 8354 was designed to retain many of the biological activities of DHEA (while eliminating its androgenic side effects) and is an even more potent inhibitor of G6PDH (K_i = 0.51 μM) (4). Many of the chemopreventive and antiproliferative effects of DHEA in rats *in vivo* can be completely reversed by supplying deoxyribonucleosides in the drinking water (16) or by i.p. injection (17), further evidence that DHEA acts by repressing DNA synthesis.

Given the difficulty of assessing the effects of a non-competitive enzyme inhibitor *in vivo*, cellular nucleotide pools are a reasonable initial surrogate for the effect of DHEA on G6PDH activity. In this study, feeding 0.3% DHEA for 4 weeks did not deplete the levels of various nucleotides in the liver, as determined by reversed phase high performance liquid chromatography coupled with diode array detection (Table III). Administration of DHEA for a longer period of time, as in the spontaneous tumorigenesis study, could conceivably have different effects on nucleotide pools. However, normal liver may not be the appropriate tissue in which to attempt to ascertain the effects of DHEA in hyperplastic cells, as it has been shown that DHEA has no apparent effect on the rate of DNA synthesis in normal epidermis at a dose that can completely suppress 12-*O*-tetradecanoylphorbol-13-acetate-stimulated mouse epidermal DNA synthesis (4). Although compound 8354 is a more potent inhibitor of G6PDH activity than DHEA, when given at half the dose it was less effective than DHEA in delaying tumorigenesis and extending lifespan (Figure 2). This less-than-predicted efficacy of compound 8354 could well be due to decreased bioavailability compared with DHEA (A.Schwartz, personal communication). Alternatively, it is possible that some of the effects of DHEA are related to the activity of a metabolite or metabolites that cannot be derived from compound 8354. The data suggesting that NAD and ATP levels may be higher in the livers of p53-deficient mice compared with those in wild-type mice (Table III) is consistent with the possibility that cells that lack p53 (and so do not undergo cell cycle arrest in response to most DNA damage; 18) consequently have lower levels of DNA repair and thus decreased consumption of these nucleotides (19).

Rather than acting through inhibition of G6PDH activity, the chemopreventive activity of DHEA and possibly of compound 8354 in this model may derive from the high frequency of lymphoma of T cell origin developed by $p53^{-/-}$ mice (8,20). Although many of the immunomodulatory activities of DHEA are classified as 'anti-glucocorticoid' (1), DHEA at the dose given in this study can itself have potent thymic effects and and can cause moderate to severe loss of thymic cortical lymphocytes and partial thinning of the medulla (J.Ward, personal communication). This effect on the thymus is rapid (with noticeable atrophy after 4 days of feeding 0.3% DHEA in the diet) and reflects changes in both apoptotic and proliferative pathways (T.Wang, S.Hursting, S.Perkins and J.Phang, submitted for publication). DHEA has been shown to inhibit lymphopoiesis selectively (21) and it appears to inhibit differentiation of prothymocytes to mature T cells (with no effect on mature lymphocytes) by affecting the thymic microenvironment (22). Our finding that DHEA lengthens lifespan in $p53^{-/-}$ mice by reliably suppressing the development of lymphoblastic lymphomas but not other types of lymphoma (Table I; 8) is consistent with such a mechanism. Whether compound 8354 works through the same mode of action is unclear: although compound 8354 tended to have the same inhibitory effect on lymphoblastic lymphoma as DHEA (albeit not to a statistically significant degree in this study; Table I and Figure 3), thymic atrophy was less common (unpublished data). The differential effects of DHEA on tumor type contrast with our previous findings in these mice that calorie restriction, while lengthening survival to a similar degree to DHEA, has no effect on the spectrum of tumors observed (7). Thus, although DHEA significantly inhibits the development of lymphoblastic lymphoma in p53-deficient mice, determining the general utility of these compounds as chemopreventive or anti-lymphomic agents (in the presence or absence of p53 tumor suppressor function) will require additional studies.

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