SPANDIDOS PUBLICATIONS

EXPERIMENTAL AND THERAPEUTIC MEDICINE 2: 221-228, 2011

Inhibitory effect of dietary atorvastatin and celecoxib together with voluntary running wheel exercise on the progression of androgen-dependent LNCaP prostate tumors to androgen independence

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Received October 26, 2010; Accepted January 7, 2011

DOI: 10.3892/etm.2011.203

Abstract. In the present study, the inhibitory effect of dietary atorvastatin, dietary celecoxib and voluntary running wheel exercise (RW) alone or in combination on the formation and growth of androgen-independent LNCaP tumors in castrated severe combined immunodeficient (SCID) mice was determined. Male SCID mice were injected subcutaneously with androgen-dependent prostate cancer LNCaP cells. When the tumors reached a moderate size, the mice were surgically castrated and treated with atorvastatin (0.02% in the diet), celecoxib (0.05% in the diet) or RW alone or in combination for 42 days. RW or celecoxib alone had a moderate inhibitory effect on the androgen-independent growth of LNCaP tumors, while atorvastatin alone had little or no effect on tumor growth. Combinations of atorvastatin and celecoxib had a stronger inhibitory effect on the formation and growth of androgen-independent LNCaP tumors than either drug alone. A combination of RW together with atorvastatin and celecoxib had the most potent inhibitory effect on the progression of LNCaP tumors to androgen-independent growth. The serum concentration of atorvastatin after 2 weeks of oral administration of atorvastatin was 6.1 ng/ml. The serum concentration of celecoxib after treatment with dietary celecoxib for 2 weeks was 1,090 ng/ml. The serum concentration of atorvastatin, but not that of celecoxib, was significantly reduced when the two drugs were given in combination. The drug concentrations observed in the animal studies are comparable or less than those commonly found in humans treated with atorvastatin or celecoxib. The results indicate that the administration of atorvastatin and celecoxib together with voluntary exercise is an effective strategy for the prevention of prostate cancer progression from androgen dependence to androgen independence.

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Key words: prostate cancer, androgen depletion, progression, exercise

Introduction

Prostate cancer is the second leading cause of cancer-related mortality among males in the US (1). Early-stage prostate cancer requires androgen for growth and thus responds to androgen- deprivation therapy (2,3). However, the disease progresses to an androgen-independent state and virtually all patients develop hormone-refractory disease. While chemotherapy options are available for patients with androgen-independent prostate cancer, these agents are only temporarily effective (4,5). Therefore, effective intervention regimens, including novel preventive agents and life-style changes that prevent the progression of androgen-dependent prostate cancer to androgen independence, are required.

A recent study, found that i.p. administration of atorvastatin and celecoxib in combination strongly inhibited the progression of androgen-dependent LNCaP tumors to androgen independence in severe combined immunodeficient (SCID) mice (6). In this study, surgical castration of SCID mice with androgen-dependent LNCaP prostate tumors caused temporary tumor regression for approximately 2 weeks followed by androgen-independent growth of the tumors. Treatment of the mice with i.p. injections of atorvastatin or celecoxib alone modestly suppressed the re-growth of LNCaP tumors after castration. A combination of lower doses of

atorvastatin and celecoxib together had a more significant effect for inhibiting the progression and growth of LNCaP tumors to androgen independence than a higher dose of either agent alone (6). A separate study found that i.p. injections of atorvastatin in combination with celecoxib inhibited the growth of androgen-independent PC-3 prostate tumors in SCID mice more than either agent alone (7). In accord with our animal data, previous epidemiological studies suggest that the use of statins (8-12) or non-steroidal anti-inflammatory drugs (NSAIDs) (13,14) are associated with a reduced risk of prostate cancer.

Although previous epidemiological studies on the association between physical activity and overall prostate cancer risk were inconclusive (15-17), recent studies suggest that physical exercise is associated with reduced risk of advanced prostate cancer and prostate cancer mortality (18-21). These results are in agreement with our studies, which found that voluntary running wheel exercise (RW) for 63 days, starting 1 week prior to the subcutaneous injection of androgen-independent PC-3 tumor cells into SCID mice, suppressed the formation and growth of the tumors (22). Mechanistic studies have shown that RW inhibited proliferation as reflected by decreased mitosis, and the exercise regimen also stimulated apoptosis as reflected by increased caspase-3 (active form) expression in the tumors. RW decreased the ratio of the percentage of mitotic cells/apoptotic cells in PC-3 tumors by 32%.

The present study assessed the inhibitory effect of dietary atorvastatin or celecoxib alone or in combination with RW on the progression of androgen-dependent LNCaP xenograft tumors to androgen independence in SCID mice. The results revealed that RW in combination with dietary atorvastatin and celecoxib had the most significnt inhibitory effect on the progression of androgen-dependent LNCaP tumors to androgen independence when compared to RW, atorvastatin or celecoxib alone, or for any of the two regimens in combination.

Materials and methods

Cells and reagents. LNCaP cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Atorvastatin and celecoxib were provided by the National Cancer Institute's Repository. Matrigel was obtained from BD Biosciences (Bedford, MA, USA). RPMI-1640 tissue culture medium, penicillin-streptomycin, L-glutamine and fetal bovine serum (FBS) were from Gibco (Grand Island, NY, USA). The LNCaP cells were maintained in RPMI-1640 culture medium containing 10% FBS that was supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (300 μg/ml). The cultured cells were grown at 37˚C in a humidified atmosphere of 5% CO_2 and passaged twice a week. Proliferating LNCaP cells at ~70% confluence were used for the animal experiment as indicated below.

Progression of androgen-dependent prostate LNCaP tumors to androgen independence in immunodeficient mice. Male SCID mice were obtained from Taconic Farms Inc. (Germantown, NY, USA). The animals were housed in sterile filter-capped microisolator cages and provided with a sterilized 5010 rodent diet (WF Fisher & Son Inc., NJ, USA) and water. LNCaP cells (2.5×10^6 cells/0.1 ml/mouse) suspended in 50% Matrigel (Collaborative Research, Bedford, MA, USA) in RPMI-1640 medium were injected subcutaneously into the right flank of the mice. After 4-6 weeks, the mice with LNCaP tumors (0.6-1.0 cm wide and 0.6-1.0 cm long) were surgically castrated to mimic antiandrogen therapy (6). The castrated mice with LNCaP tumors were treated with AIN76A diet containing 0.02% atorvastatin, AIN76A diet containing 0.05% celecoxib or RW alone or in combination. The mice treated with RW had free access to the wheel 24 h/day for the whole treatment period (42 days). The running wheels were connected with digital counters to measure the running wheel revolutions (22). Tumor size (length x width; cm^2) and body weight were measured once every third day following surgical castration. The development of androgen independence was monitored by the growth of tumors. The animal experiment was carried out under an Institutional Animal Care and Use Committee (IACUC)-approved protocol.

Serum levels of celecoxib, atorvastatin and their metabolites. Serum samples (100 μl each) were treated with 10 μl of 5% ascorbic acid prior to being stored at -70˚C. The extraction of celecoxib and atorvastatin from the serum samples was carried out by treatment with 100 μl of 0.4 mol/l sodium phosphate buffer (pH 5.0), followed by shaking with 1,000 μl of methyl-tert-butyl ether. Following centrifugation, the methyl-tert-butyl ether extract was transferred to another tube and evaporated to dryness. The aqueous residues were dried and consecutively extracted with 1,000 μl of ethyl acetate. The ethyl acetate extract was combined with the dried methyl-tert-butyl ether extract and dried. The residue was reconstituted in 100 μl of acetonitrile/water (1:1) and the sample was centrifuged. The resulting supernatant (20 μl) was injected into a liquid chromatography tandem mass spectrometry system (LC-MS/MS). The absolute solvent extraction recoveries of celecoxib (1-4,100 ng/ml) and atorvastatin (1-4,100 ng/ml) from serum were 60-67% and 70-75%, respectively.

For drug and metabolite analysis, LC/MS was performed on a Thermo LTQ linear ion trap mass detector (ThermoFisher Scientific) interfaced with an electrospray ionization probe to a Surveyor HPLC system (Thermo Fisher Scientific, Pittsburg, PA, USA) equipped with a refrigerated (4˚C) autosampler. Chromatographic separation was carried out on a Phenomenex Gemini C18 column (50x2.0-mm i.d., 3 μm particle size). The LC mobile phases consisted of acetonitrile/water [10:490 (v/v)], containing 0.2 mmol/l formic acid (solvent A) and acetonitrile/water [450:50 (v/v)], containing 0.2 mmol/l formic acid (solvent B). The mobile phase was delivered at 0.2 ml/min. For the 7-29 min following the injection of the extracted drugs in solvent B:A (20:80), the column was eluted with a linear gradient from B:A (20:80) to B:A (70:30) and then with B:A (70:30) from 29-34 min before re-equilibration with B:A (20:80) for 8 min prior to the injection of the next sample. The LC eluent flow after 2 min was introduced into the mass spectrometer for data acquisition. The MS/MS parameters in the negative-ion mode were tuned to maximize the generation of deprotonated drug molecules. The data acquired were processed by Xcalibur software (version 2.0, ThermoFisher, Thermo Electron). Celecoxib and atorvastatin standards in control serum were analyzed alongside the experimental samples and were used for the calculation of serum levels.

Since authentic metabolite standards were not available, celecoxib was used as a surrogate standard for the metabolites of celecoxib and atorvastatin for the metabolites of atorvastatin. Therefore, the reported levels of metabolites are estimated values. The identification of metabolites is described later.

Statistical analysis. The analysis of percent change in tumor size from baseline was based on a repeated measurement model (23). Heterogeneous autoregressive correlation was used to account for the within-mice correlation. The analysis of variance (ANOVA) model was used to analyze the percentage of change from the baseline for tumor size at day 42 (last time point). Bonferroni adjustment was used for the comparison of the triple-treatment regimen with any of the double-treatment regimens as well as the comparison of double-treatment regimens with any of the single-treatment regimens (nine comparisons). ANOVA with Tukey-Kramer multiplicity adjustment was used for the comparison of body weight and food and drinking fluid consumption in the various groups. An overall significance level of 5% was used for the multiple tests.

Results

Inhibitory effect of dietary atorvastatin, dietary celecoxib and voluntary running wheel exercise on androgen-independent growth of LNCaP tumors in castrated SCID mice. The male SCID mice were injected subcutaneously with androgen-dependent prostate cancer LNCaP cells as described in Fig. 1. When the tumors reached a moderate size (0.6-1.0 cm wide and 0.6-1.0 cm long), the mice were assigned into eight groups. The mice in group 1 were fed the regular AIN76A diet, the mice in group 2 were fed the AIN76A diet containing 0.02% atorvastatin, the mice in group 3 were fed the AIN76A diet containing 0.05% celecoxib, the mice in group 4 were fed the regular AIN76A diet and placed in a cage equipped with a running wheel, the mice in group 5 were placed in a cage equipped with a running wheel and fed the AIN76A diet containing 0.02% atorvastatin, the mice in group 6 were placed in a cage equipped with a running wheel and fed the AIN76A diet containing 0.05% celecoxib, the mice in group 7 were fed the AIN76A diet containing both 0.02% atorvastatin and 0.05% celecoxib and the mice in group 8 were placed in a cage equipped with a running wheel and fed the AIN76A diet containing both 0.02% atorvastatin and 0.05% celecoxib. Each group had 5 mice with the exception of groups 7 and 8 which had 4 mice.

As shown in Fig. 1A, the LNCaP tumors in the groups regressed initially in response to castration, but the tumors then progressed to androgen-independence and started to grow at 2-4 weeks post-castration. Regrowth of the tumors started at 15 days post-castration in the control group and at 18 days post-castration in the atorvastatin, celecoxib or RW group. Regrowth of the tumors in the atorvastatin + celecoxib, atorvastatin + RW, celecoxib + RW or the atorvastatin + celecoxib + RW group started at 18, 21, 15 and 21 days post-castration, respectively (Fig. 1A). The time that it took for the tumors to reach their original size at the time of castration in the control, atorvastatin, celecoxib or RW groups was 24, 27, 27 and 30 days, respectively (Fig. 1A). The time that it took for

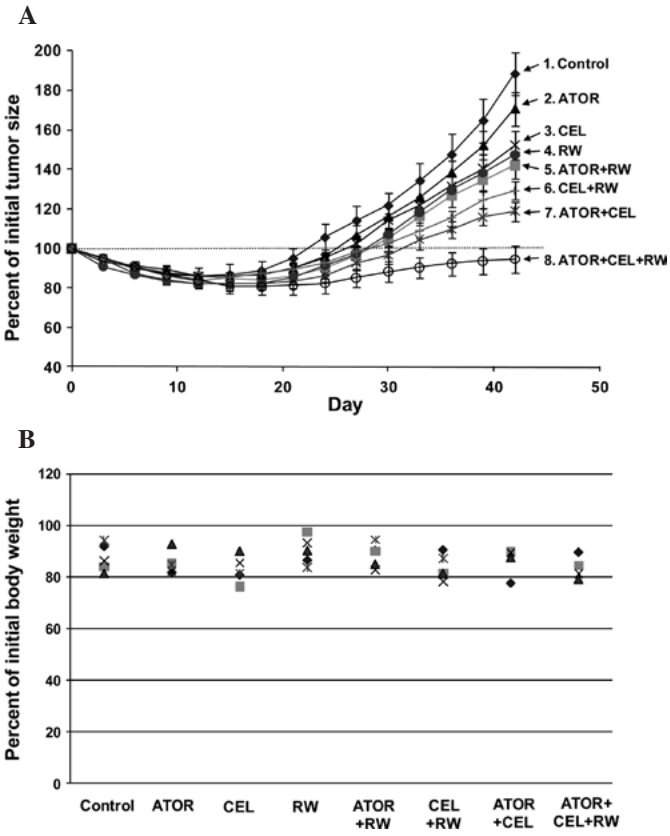

Figure 1. Effects of atorvastatin (ATOR), celecoxib (CEL) and running wheel exercise (RW) alone or in combination on the androgen-independent growth of LNCaP tumors and body weight of severe combined immunodeficient (SCID) mice. The male SCID mice (5 mice/group in groups 1-6; 4 mice/group in groups 7-8) were injected subcutaneously with LNCaP cells in 50% Matrigel (2.0×10^6 cells/0.1 ml). After 4-6 weeks, mice with LNCaP tumors (0.6-1.0 cm wide and 0.6-1.0 cm long) were surgically castrated. The castrated mice were treated with atorvastatin (0.02% in the diet), celecoxib (0.05% in the diet) or RW alone or in combination for 42 days. (A) Tumor size (length x width; cm^2) was measured and expressed as percentage of the initial tumor size. (B) Body weights (g) were measured and expressed as a percentage of the initial body weight.

the tumors to reach their original size at the time of castration in the atorvastatin + celecoxib, atorvastatin + RW, celecoxib + RW or the atorvastatin + celecoxib + RW group was 33, 30, 30 and >42 days, respectively (Fig. 1A). The growth rate in percent change in tumor size from the baseline for the atorvastatin + celecoxib + RW group was significantly smaller than that for any other group ($p \leq 0.0214$).

RW or administration of celecoxib alone had a moderate inhibitory effect on the androgen-independent growth of LNCaP tumors, while administration of atorvastatin alone had little or no effect on the tumors (Fig. 1A). A combination of atorvastatin and celecoxib had a stronger inhibitory effect on the growth of androgen-independent LNCaP tumors than either treatment alone (Fig. 1A). Treatment with a combination of RW together with atorvastatin and celecoxib had the most potent inhibitory effect on the androgen-independent growth of LNCaP tumors (Fig. 1A). The ANOVA model with Bonferroni adjustments were used to compare the percentage of the initial tumor size between the treatment groups. The percentage of the initial tumor size at day 42 after treatment in the atorvastatin + celecoxib group was significantly smaller

Table I. Food and fluid consumption in SCID mice treated with atorvastatin, celecoxib or RW alone or in combination.

Group	No. of mice	Food consumption (g/mouse/day)	Fluid consumption (ml/mouse/day)	Running distance (miles/mouse/day)
Control	5	4.31±0.12	5.52±0.08	-
Atorvastatin	5	3.99±0.11	5.47±0.11	-
Celecoxib	5	3.86±0.12	5.53±0.13	-
RW	5	5.39±0.13	6.22±0.11	1.31±0.22
Atorvastatin + RW	5	4.10±0.14	6.03±0.14	1.29±0.23
Celecoxib + RW	5	4.02±0.11	5.98±0.16	1.32±0.14
Atorvastatin + Celecoxib	4	3.98±0.14	5.63±0.11	-
Atorvastatin + Celecoxib + RW	4	4.08±0.20	6.04±0.12	1.28±0.26

Male severe combined immunodeficient (SCID) mice were injected subcutaneously with LNCaP cells in Matrigel. After 4-6 weeks, mice with LNCaP tumors (0.6-1.0 cm wide and 0.6-1.0 cm long) were surgically castrated and treated with atorvastatin (0.02% in the diet), celecoxib (0.05% in the diet) and running wheel exercise (RW) alone or in combination for 6 weeks. Wheel revolutions and the consumption of food and fluid were recorded. Each value represents the mean ± SE. Statistical analysis was performed using ANOVA with the Tukey-Kramer multiplicity adjustment (adjusted p-values were used in the following). For food consumption, the difference between the RW group and any other group was statistically significant ($p<0.001$). The differences in food consumption between the atorvastatin group and the atorvastatin + RW group, between the celecoxib group and the celecoxib + RW group, and between the atorvastatin + celecoxib group and the atorvastatin + celecoxib + RW group were not statistically significant ($p>0.05$). Statistically significant effects on fluid consumption were: control vs. RW, $p<0.01$; atorvastatin vs. RW, $p<0.01$; atorvastatin vs. atorvastatin + RW, $p<0.05$; atorvastatin vs. atorvastatin + celecoxib + RW, $p<0.05$; celecoxib vs. RW, $p<0.01$; atorvastatin + celecoxib vs. RW, $p<0.05$. For running distance, the difference between any two groups was not statistically significant ($p>0.05$).

than the atorvastatin group or the celecoxib group ($p\leq0.0026$). The percentage of the initial tumor size at day 42 after treatment in the atorvastatin + RW group was significantly smaller than in the atorvastatin group ($p=0.0086$). The percentage of the initial tumor size at day 42 after treatment in the celecoxib + RW group was significantly smaller than in the celecoxib group ($p=0.041$). The percentage of the initial tumor size at day 42 after treatment in the atorvastatin + celecoxib + RW group was significantly smaller than in any of the two-regimen combination groups ($p\leq0.032$).

The average distances ± SE the mice ran on the running wheel were 1.31±0.22, 1.29±0.23, 1.32±0.14 and 1.28±0.26 miles/mouse/day in the RW, atorvastatin + RW, celecoxib + RW and atorvastatin + celecoxib + RW groups, respectively (Table I). The difference in miles run per mouse between any two groups was not statistically significant ($p>0.05$). The RW group consumed 25% more food and 13% more water compared to mice in the control group (Table I). The difference in food consumption between the atorvastatin group and the atorvastatin + RW group, between the celecoxib group and the celecoxib + RW group, and between the atorvastatin + celecoxib group and the atorvastatin + celecoxib + RW group was not statistically significant ($p>0.05$; Table I). The results indicate that RW did not significantly alter the intake of atorvastatin and/or celecoxib. The effect of the various treatments on body weight is described in Fig. 1B. The mean ± SE for the percentage of the initial body weight after 42 days of treatment was 87.6±5.4 for the control group, 85.4±4.3 for the atorvastatin group, 82. ±5.2 for the celecoxib group, 90.3±5.4 for the RW group, 86.1±5.8 for the atorvastatin + celecoxib group, 88.6±4.7 for the atorvastatin + RW group, 83.8±5.1 for the celecoxib + RW group and 83.7±4.6 for the atorvastatin + celecoxib + RW group. Statistical analysis with the Tukey-Kramer multiple comparison test showed that the difference in the percentage of the initial body weight between any two groups was not statistically significant ($p>0.05$).

Serum levels of atorvastatin and celecoxib and metabolite identification in SCID mice

Serum levels. The serum levels of atorvastatin and celecoxib were determined in order to show the levels associated with biological activity in our animal model. The serum concentration of atorvastatin after 2 weeks of oral administration (0.02% in diet) was 6.1 ng/ml (Table II). Fig. 2A shows the HPLC chromatograms of serum samples after oral administration of celecoxib and atorvastatin in mice, and Fig. 2B shows the fragmentation patterns of $[M-H]^-$ from celecoxib, atorvastatin and their major metabolites. Two atorvastatin metabolites, *p*-hydroxy-atorvastatin and *o*-hydroxy-atorvastatin, were also tentatively identified and quantified (Fig. 3). As shown in Table II, the serum level of *p*-hydroxy-atorvastatin was 6.28 ng/ml and that of *o*-hydroxy-atorvastatin was 22.6 ng/ml after 2 weeks of oral administration of atorvastatin (0.02% in the diet). The serum concentration of celecoxib after treatment with celecoxib (0.05% in the diet) for 2 weeks was 1,090 ng/ml (Table II). After 2 weeks of oral administration of celecoxib, the serum levels of the hydroxy-celecoxib and carboxy-celecoxib metabolites were 235 and 331 ng/ml, respectively (metabolite identification described below). In mice treated with dietary atorvastatin + celecoxib, the serum levels of atorvastatin and its metabolites were significantly lower than those in mice treated with atorvastatin alone, while the serum levels of celecoxib and its metabolites were similar to those from mice treated with celecoxib alone (Table II).

Metabolite identification. To identify metabolites using LC-MS, the characterization of chromatographic and mass spectrometric properties of candidate compounds were compared to those of the parent compounds and other likely

Table II. Average daily level of celecoxib, atorvastatin and their metabolites in serum from SCID mice treated with celecoxib or atorvastatin alone or in combination.

Treatment	Serum concentration (ng/ml)					
	Atorvastatin	*p*-Hydroxy-atorvastatin	*o*-Hydroxy-atorvastatin	Celecoxib	Hydroxy-celecoxib	Carboxy-celecoxib
Atorvastatin	6.12±2.26	6.28±2.44	22.6±6.9	-	-	-
Celecoxib	-	-	-	1090.4±127.9	234.6±26.8	331.1±58.4
Atorvastatin + celecoxib	0.45±0.30	2.03±1.99	10.3±2.5	963.2±69.8	274.8±15.8	323.9±52.4

Male severe combined immunodeficient (SCID) mice (10/group) were fed AIN76A diet containing 0.05% celecoxib, 0.02% atorvastatin or 0.05% celecoxib + 0.02% atorvastatin for 2 weeks. Serum samples were collected at 7 am (5 mice) and 7 pm (5 mice) on the last day of the experiment and analyzed for the levels of celecoxib, atorvastatin and their metabolites. Since large differences in drug levels at 7 am and 7 pm were not observed, the data from the animals in each group were combined. Values for the mean ± SE from 10 animals in each group are shown.

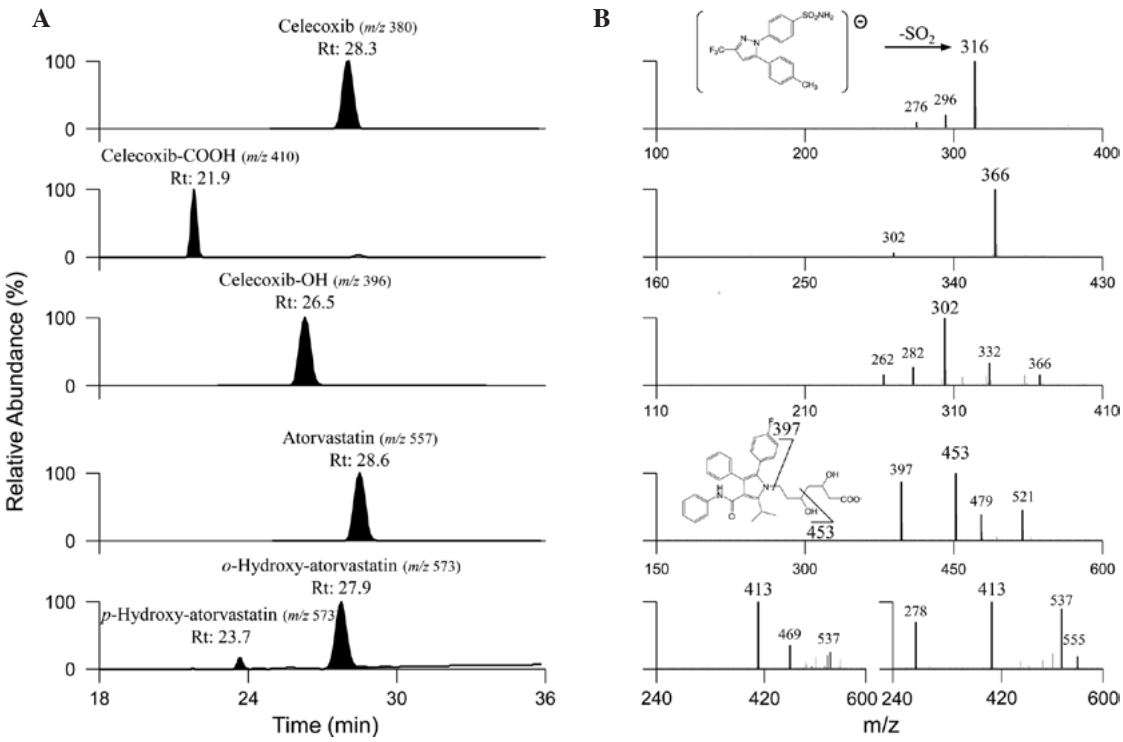

Figure 2. Celecoxib, atorvastatin and their metabolites in mouse serum. Male severe combined immunodeficient mice (10/group) were fed the AIN76A diet containing 0.05% celecoxib, 0.02% atorvastatin or 0.05% celecoxib + 0.02% atorvastatin for 2 weeks. Serum samples were collected on the last day of the experiment and analyzed for the levels of celecoxib, atorvastatin and their metabolites. (A) HPLC chromatogram of the parent compounds and metabolites. (B) MS^n spectra and structural elucidation of deprotonated ions from celecoxib, atorvastatin and their metabolites.

metabolites. Their fragmentation patterns were analyzed based on the MS^n fragmentation of the major product ions (Fig. 2B). Moreover, the MS spectra obtained from the samples tested were compared to known control samples (parent drug), so that possible metabolites could be identified.

In this study, the negative-ion ESI mode was more sensitive for the analysis of celecoxib and atorvastatin than the positive-ion ESI mode. The deprotonated ion at *m/z* 380 for celecoxib with a retention time of 28.2 min generated minor product ions of *m/z* 296 and 276, as well as a major product ion of *m/z* 316, designated as the pathway shown in Fig. 2B. The product ions at *m/z* 296 and 276 were generated by two sequential losses of 20 (HF) from the product ion at *m/z* 316. The product ion at *m/z* 316 originates from the $[M-H]^-$ ion by the loss of 64 (SO_2). Two peaks eluted earlier at 21.9 and 26.5 min showed deprotonated ions of *m/z* 410 and 396, which were 30 and 16 Da higher, respectively, than that of the parent compound celecoxib, indicating that they were carboxylated and monohydroxylated metabolites of celecoxib. The CID product ion spectrum of the ion at *m/z* 410 showed a minor product ion at *m/z* 302 $[(M-H-CO_2-SO_2)^-]$ and a major product ion at *m/z* 366 $[(M-H-CO_2)^-]$. Based on these data, the metabolite was identified as carboxy-celecoxib (Fig. 3). The CID product ion spectrum of the ion at *m/z* 396 showed minor

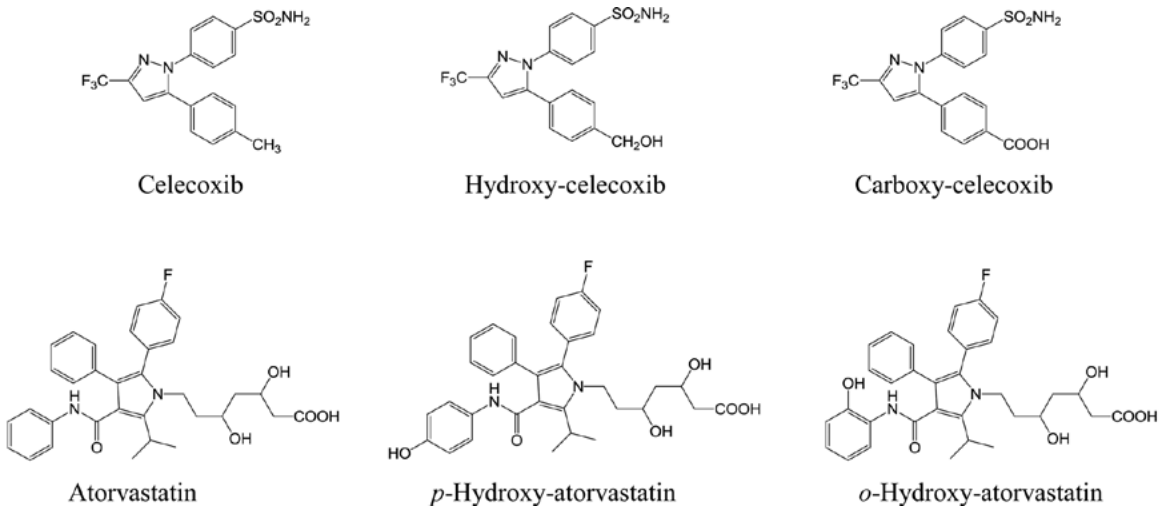

Figure 3. Structures of atorvastatin, celecoxib and their metabolites.

product ions at *m/z* 366 [(M-H-CH_2O)$^-$], 332 [(M-H-SO_2)$^-$], 282 [(M-H-CH_2O-SO_2-HF)$^-$] and 262 [(M-H-CH_2O-SO_2-2HF)$^-$], as well as a major product ion at *m/z* 302 [(M-H-CH_2O-SO_2)$^-$]. The loss of 30 (CH_2O) suggested that the hydroxylation occurred on the methyl moiety of the 5-(4-methyl) phenyl group. Based on these data, the metabolite was identified as a hydroxy-methyl metabolite of celecoxib (Fig. 3). Carboxy-celecoxib and hydroxy-celecoxib were shown to be the major celecoxib metabolites in the mouse serum samples, which was consistent with a previous report on rabbit blood samples (24).

The deprotonated ion at *m/z* 557 for atorvastatin with a retention time of 28.6 min (Fig. 2A) generated minor product ions of *m/z* 521 [(M-H-2H_2O)$^-$] and 479, as well as major product ions of *m/z* 453 and 397, designated as a pathway shown in Fig. 2B. Cleavage of the side-chain produced product ions of *m/z* 479, 453 and 397. The ion at *m/z* 479 is generated by the loss of water and $C_2H_4O_2$ from the molecule. The ion at *m/z* 453 was generated by the loss of 104 ($C_4H_8O_3$). The ion at *m/z* 397 was generated from the cleavage of the side-chain between the pyrrole nitrogen and C-7 of the side-chain, by the loss of the heptanoic acid side-chain, $C_7H_{12}O_4$. Two peaks eluted earlier at 23.7 and 27.9 min showed a deprotonated ion of *m/z* 573, which was 16 Da higher than that of the parent compound atorvastatin, suggesting that they were both mono-hydroxylated metabolites.

The MS^2 spectrum of the first peak displayed major product ions of *m/z* 413 [(M-H-$C_7H_{12}O_4$)$^-$] and minor product ions of *m/z* 469 [(M-H-$C_4H_8O_3$)$^-$], 537 [(M-H-2H_2O)$^-$] and 555 [(M-H-H_2O)$^-$]. The MS^2 spectrum of the second peak displayed major product ions of *m/z* 278 ($C_{19}H_{17}FN^-$), 413 [(M-H-$C_7H_{12}O_4$)$^-$] and 537 [(M-H-2H_2O)$^-$], and a minor product ion of *m/z* 555 [(M-H-H_2O)$^-$]. The mass difference between the multiple product ions at *m/z* 537, 469, 413 generated from the metabolites and the respective equivalent product ions at *m/z* 521, 453, 397 from atorvastatin was 16 Da, suggested that hydroxylation did not occur on the dihydroxyhepanoic acid moiety, and the fragmentation pathways for the metabolites were similar to that of atorvastatin. There are three possible sites for hydroxylation, ortho-, meta- and para-positions on each of the aromatic rings. Based on a previous report (25) and their retention times, our metabolites are *p*-hydroxy-atorvastatin and *o*-hydroxy-atorvastatin, as shown in Fig. 3. Both are pharmacologically active (25,26).

Discussion

In the present study, we found that the triple combination of RW together with dietary administration of atorvastatin and celecoxib was highly effective at inhibiting the progression and growth of androgen-dependent LNCaP prostate tumors to androgen independence in castrated SCID mice (Fig. 1A). Administration of atorvastatin and celecoxib had a stronger inhibitory effect on the growth of LNCaP tumors than either drug alone. RW significantly increased the inhibitory effect of atorvastatin or celecoxib on the growth of LNCaP tumors. Treatment with RW + atorvastatin + celecoxib had the most significant inhibitory effect on the formation and growth of androgen-independent LNCaP tumors (Fig. 1A). We anticipate that this triple treatment regimen will also be effective at inhibiting the formation and growth of other cancers. To the best of our knowledge, the present study is the first example of the use of two drugs and exercise in combination for cancer prevention.

Previous studies have shown that administration of a combination of atorvastatin and celecoxib was more effective than atorvastatin or celecoxib alone for inhibiting the formation of azoxymethane-induced colon cancer in rats (27), the growth of androgen-independent PC-3 tumors in SCID mice (7) and the progression and growth of androgen-dependent LNCaP tumors to androgen independence in castrated SCID mice (6). In earlier studies, RW inhibited the formation of chemically induced colon and breast cancer in rats (28-30), UVB-induced skin cancer in mice (31), as well as the formation and growth of androgen-independent PC-3 prostate tumors and Panc-1 pancreatic tumors growing as xenografts in SCID mice (22). In one of these studies, exercise enhanced apoptosis in the tumors (22). The available evidence suggests that voluntary exercise has an anti-inflammatory effect (32,33).

In the present study, we found that oral administration of 0.02% atorvastatin in the AIN76A diet to male SCID mice

for 2 weeks resulted in a serum concentration of 6.1 ng/ml (Table II). An earlier study showed that oral administration of atorvastatin (20 mg) in humans resulted in a peak plasma level of ~7 ng/ml (34). After oral administration of atorvastatin (20 mg) once a day for 14 days to humans, the peak plasma level was 15 ng/ml (35). It was also reported that oral administration of celecoxib (200 mg) to humans resulted in a peak plasma level of 600-1,300 ng/ml (36). In the present study, oral administration of celecoxib (0.05% in the AIN76A rodent diet) for 2 weeks in male SCID mice resulted in a plasma level of 1,090 ng/ml. The dramatic lowering of the serum level of atorvastatin and the somewhat smaller lowering of the levels of its metabolites in mice that received celecoxib in combination with atorvastatin for 2 weeks compared to atorvastatin alone suggests that celecoxib administration enhanced the metabolism of atorvastatin and its metabolites. The serum levels of celecoxib and atorvastatin in the present study in male SCID mice were comparable to or lower than those observed in humans. Our results indicate that the serum levels of atorvastatin and celecoxib associated with preventive efficacy on the progression of prostate tumors to androgen independence in the SCID mouse model are achievable in humans.

In summary, the results of the present study demonstrate that the triple combination of RW combined with oral administration of atorvastatin and celecoxib (regimens that likely work by different mechanisms) has a potent inhibitory effect on the progression and growth of androgen-dependent prostate tumors to androgen independence in a xenograft model in SCID mice. The serum levels of atorvastatin and celecoxib in the present study were comparable to or lower than the levels obtained in patients taking these drugs. The results of our study warrant a clinical trial to determine the effect of a combination of exercise, atorvastatin and celecoxib on the progression and growth of androgen-dependent prostate tumors to androgen independence in prostate cancer patients, as well as to determine the effect of the triple regimen on the progression and growth of other cancers.

Acknowledgements

The present study was supported by NIH grants CA121391 and CA133902. The authors thank Ms. Annette Dionisio for her excellent help in the preparation of this manuscript.

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