Physical Activity and Breast Cancer Risk in a Cohort of Young Women

Beverly Rockhill, Walter C. Willett, David J. Hunter, JoAnn E. Manson, Susan E. Hankinson, Donna Spiegelman, Graham A. Colditz*  

Background: Increased physical activity has been hypothesized to be a means of primary prevention of breast cancer. We examined the associations between physical activity at two different times in life and breast cancer risk. Methods: We analyzed data from the Nurses’ Health Study II, a prospective study of women aged 25–42 years in 1989. On the baseline survey, women were asked, “While in high school and between the ages 18 and 22 years, how often did you participate in strenuous physical activity at least twice a week?” We averaged answers to these two questions to develop a measure of late adolescent activity. Women were also asked at baseline to report the number of hours per week they currently spent in different nonoccupational activities. During 6 years of follow-up, we identified 372 cases of invasive breast cancer. Data were analyzed by use of multivariate pooled logistic regression to produce relative risk (RR) and confidence intervals (CIs) of being diagnosed with the disease. Results: Women who were more active in late adolescence were not at reduced risk of breast cancer compared with less active women. For those women who reported engaging in strenuous activity at least twice per week for 10–12 months per year in late adolescence, the RR of cancer, compared with those who never engaged in such activity, was 1.1 (95% CI = 0.8–1.6). Similarly, higher levels of recent nonoccupational physical activity were not associated with reduced risk of breast cancer (RR for ≥7 hours of activity/week relative to <1 hour/week = 1.1; 95% CI = 0.8–1.5). Conclusion: Our findings do not support a link between physical activity, in late adolescence or in the recent past, and breast cancer risk among young adult women. [J Natl Cancer Inst 1998;90:1155–60]  

Increased physical activity has been hypothesized to be a means of primary prevention of breast cancer. Activity can lead to lower cumulative exposure to circulating ovarian hormones; specifically, strenuous physical activity at a young age delays the onset of regular ovulatory cycles (1–4), and activity during the reproductive years may reduce levels of circulating ovarian hormones and the frequency of regular cycles (5,6). Physical activity might also act to lower breast cancer risk among postmenopausal women by reducing fat stores, which are the locus of conversion of the androgen androstenedione to estrone (7,8).

There is currently no scientific consensus on the critical time period of exposure or on the level of intensity of activity that is needed to influence breast cancer risk. Consequently, epidemiologic studies of physical activity and breast cancer have been heterogeneous in terms of the age at breast cancer diagnosis, methods for measuring intensity and duration of activity, and the period in life for which activity was assessed. For instance, Bernstein et al. (9) conducted a case–control study of women under age 40 years and quantified physical activity as categories of average number of hours per week of participation in physical exercise between menarche and the study reference date. To date, Bernstein et al. have reported the strongest reduction in risk associated with physical activity, and they concluded from their analyses that lifelong activity pattern is the critical exposure of interest. Most recently, Thune et al. (10), in a 14-year prospective study of women aged 20–54 years at baseline, considered both time and intensity in their activity classification. Thune et al. found that breast cancer risk was reduced with higher levels of recent activity and that the effect of physical activity on breast cancer risk appeared stronger in premenopausal than in postmenopausal women, although the number of premenopausal cases (100) was relatively small.

To date, Bernstein et al. (9) have been the only investigators to consider a measure of lifelong activity. Several investigators have focused on activity in late adolescence/early adulthood (e.g., 11–13), while others (14–16), including Thune et al. (10), examined only adult activity. Still other investigators (17–19) examined both time periods.

Perhaps because of such heterogeneity in methods and because selection and recall biases could influence the results of case–control studies, the epidemiologic evidence for the association between physical activity and breast cancer risk is inconsistent. Some studies have found a strong to moderate reduction in risk associated with higher levels of physical ac-

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See “Notes” following “References.”
activity (9–11,17), others found only slight decreases in risk (14,16), and in others no association (18,19) or even the suggestion of a positive association (15,16) was observed.

Here, we report the associations between nonoccupational physical activity and risk of breast cancer in a large prospective cohort of mainly premenopausal women. We examine the effect of activity in late adolescence as well as recent adult activity.

Methods

The Nurses’ Health Study II is a prospective cohort study that was established in 1989, when 116,671 nurses, 25–42 years of age, completed a baseline questionnaire about their medical histories and lifestyles. Subsequent questionnaires requesting updated information on risk factors and medical events were mailed every 2 years. All members of the cohort were free of cancer, except for nonmelanoma skin cancer, in 1989. We observed 95% of the potential person-time of follow-up in this cohort between 1989 and 1995 (618,010 of 651,080 person-years); the remaining 5% of the person-years were lost to follow-up. The protocol for the study was approved by the Human Research Committees of the Brigham and Women’s Hospital and the Harvard School of Public Health, Boston, MA.

Assessment of Physical Activity

On the 1989 questionnaire, nurses were asked two questions pertaining to physical activity earlier in their lives. They were asked to report the number of months per year that they engaged in strenuous physical activity (e.g., swimming, aerobics, field hockey, basketball, cycling, and/or running) at least once per week of moderate plus vigorous activity into five levels. The reference group were those women with less than 1 hour of moderate plus vigorous physical activity per week. The remaining categories were 1.0–1.9, 2.0–3.9, 4.0–6.9, and greater than or equal to 7.0 hours of moderate plus vigorous physical activity per week. Again, we modeled these categories as a series of four indicator variables.

Data on Menstrual Regularity and Body Size Parameters

In 1989, nurses were asked several questions about their menstrual cycles earlier in life. They reported the usual length of their menstrual cycle during ages 18–22 years (<21, 21–25, 26–31, 32–39, 40–50, and >50 days, or too irregular to estimate) and the pattern of their menstrual cycles (very regular, regular, usually irregular, always irregular, or no periods) during both high school and ages 18–22 years (while not using oral contraceptives).

In 1989, women reported their height, current weight, and weight at age 18 years. We used body mass index (BMI, weight in kilograms divided by height in meters squared) as a measure of adiposity. We also calculated weight change during the period between age 18 years and 1989. The self-reported measure of weight at age 18 years has been validated in a subsample of 118 women (20) through review of college entrance physical examination records. The Spearman correlation between recalled and recorded weight at age 18 years was .87. Participants slightly underreported weight at age 18 years (mean difference, 1.4 kg). The precision of self-reported current weight was evaluated in a subsample of 140 participants from a similar cohort study of nurses (21). Trained technicians visited the subsite participants twice, approximately 6 months apart, to measure weight. The Pearson correlation coefficient between self-reported weight and the average of the technicians’ two measurements was .97. Again, participants underreported current weight; the mean difference was 3.3 kg. The degree of underreporting did not vary appreciably across levels of current weight.

Data on Other Risk Factors

Age at menarche was reported on the baseline questionnaire. Information on other risk factors, including parity, age at first birth, history of benign breast disease, family history of breast cancer in mother and/or sister, and oral contraceptive use, was reported on the baseline questionnaire and was updated every 2 years based on responses to the follow-up questionnaires. Thus, women’s status on these covariates could change over time. We also included data on current alcohol consumption (within the past year), as measured on the 1989 baseline questionnaire, as a covariate in models.

On each questionnaire, we collected data on menopausal status and, where applicable, age at menopause. Postmenopausal women were asked about their use of hormone replacement therapy on each survey. At baseline, only 2.4% of the women were postmenopausal; this percentage rose to 4.6% by 1993. Less than 5% of the breast cancer cases in these analyses were diagnosed postmenopausally. The descriptive data and associations we report here (based on the sample containing both premenopausal and postmenopausal women, with no adjustment for menopausal status or postmenopausal hormone use) were changed little when we restricted the sample to women premenopausal at each time point or when we used the entire sample but included the covariates of menopausal status and postmenopausal hormone use in the statistical models.

Identification of Invasive Breast Cancer Cases

On the 1991, 1993, and 1995 questionnaires, women were asked if they had been diagnosed with breast cancer in the previous 2 years. (Women who missed a questionnaire, but re-entered the cohort on a subsequent questionnaire, reported about breast cancer diagnoses since the last questionnaire they returned.) Deaths in the cohort are reported by family members and the postal service or were detected by a search of the National Death Index (for participants who have been lost to follow-up. In this cohort, there have been 19 deaths from breast cancer; two of these deaths were detected by the National Death Index search. For identified cases of breast cancer, we requested permission from each case subject to obtain hospital records and pathology reports. Pathology reports were obtained for 89% of the cases subjects, and of these, 98% confirmed the self-reported diagnosis of breast cancer. Those reported cases whose records failed to confirm breast cancer were excluded from analyses. However, because the degree of self-reporting accuracy was high, we included self-reported cases from whom records could not be obtained. There were a total of 372 case subjects with invasive breast cancer for whom we obtained complete data on the exposure(s) and covariates of interest. All results reported here were unchanged when we included in situ cases (n = 40) with the invasive cases.

Statistical Analysis

We used multivariate pooled logistic regression to model the risk of being diagnosed with breast cancer over the 6-year follow-up period (1989 through 1995) for each of the activity variables. Women were classified with respect to all potential confounders and then with respect to their physical activity level. Each participant contributed person-time of follow-up from the time the baseline questionnaire was returned in 1989 until the end of follow-up (June 1, 1995), the onset of the outcome of interest, death from any cause, or loss to follow-up. The number of cases and person-years that occurred within each exposure level within each strata were counted. For covariates that remain constant throughout the duration of the study, such as age at menarche, cases and person-time of follow-up were assigned to the exposure level observed at baseline in 1989. For time-varying covariates, such as current oral contraceptive use or parity, cases and person-time were re-assigned every 2 years according to the updated exposure values reported on each of the
Characteristics of participants (as reported on 1989 baseline questionnaire) according to average level of vigorous physical activity during high school and between ages 18–22 years*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Average level of vigorous physical activity during high school and between ages 18–22 years*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Mean age (standard deviation)</td>
<td>36.0 (4.3)</td>
</tr>
<tr>
<td>Menarche (14 y of age %)</td>
<td>15.6</td>
</tr>
<tr>
<td>Nulliparas (%)</td>
<td>25.7</td>
</tr>
<tr>
<td>Current oral contraceptive use (%)</td>
<td>9.0</td>
</tr>
<tr>
<td>Never used oral contraceptives (%)</td>
<td>17.2</td>
</tr>
<tr>
<td>History of benign breast disease (%)</td>
<td>30.9</td>
</tr>
<tr>
<td>History of breast cancer in mother and/or sister (%)</td>
<td>6.3</td>
</tr>
<tr>
<td>Never smokers (%)</td>
<td>72.0</td>
</tr>
<tr>
<td>No recent alcohol consumption (%)</td>
<td>29.4</td>
</tr>
<tr>
<td>Irregular menstrual cycles in high school (%)</td>
<td>29.6</td>
</tr>
<tr>
<td>Average menstrual cycle length &gt;40 days</td>
<td>8.6</td>
</tr>
<tr>
<td>between ages 18 and 22 y (%)</td>
<td></td>
</tr>
<tr>
<td>Body mass index &gt;23.0 at age 18 y (%)</td>
<td>25.2</td>
</tr>
<tr>
<td>Body mass index &gt;25.0 in 1989 (%)</td>
<td>33.4</td>
</tr>
<tr>
<td>Weight gain &gt;10 kg, age 18 y to 1989 (%)</td>
<td>31.9</td>
</tr>
<tr>
<td>Height &gt;67 inches (%)</td>
<td>13.4</td>
</tr>
</tbody>
</table>

*Participation in strenuous physical activity or sports at least twice per week.
associated with either late adolescent or recent physical activity level.

In age-adjusted analyses, the level of late adolescent physical activity was not associated with an increased risk of breast cancer in this cohort (Table 3). Adjustment for a variety of measured breast cancer risk factors changed the age-adjusted RRs little; all RRs remained close to a value of 1.0. When we examined the two separate physical activity variables that were averaged to derive the late adolescent physical activity variable (strenuous activity during high school and between ages 18 and 22 years), we found results nearly identical to those in Table 3. We investigated the possibility that the relationship between late adolescent physical activity and the risk of breast cancer might vary according to parity (nulliparous versus parous), family history of breast cancer, history of benign breast disease, BMI at age 18 years (in tertiles), BMI in 1989 (in tertiles), and amount of weight change between age 18 years and 1989 (in tertiles). We found no meaningful differences in the association between late adolescent activity and breast cancer risk across strata of any of these variables (data not shown).

In age-adjusted analyses, average weekly hours of moderate plus vigorous physical activity (see Assessment of Physical Activity in “Methods”) in 1989 was also not associated with breast cancer risk (Table 4). Again, all RRs were close to a value of 1.0 and were unchanged by adjustment for measured breast cancer risk factors as well as level of late adolescent physical activity. We similarly found no association between breast cancer risk and average weekly hours of total physical activity (light plus moderate plus vigorous) in 1989 or between risk and hours of vigorous activity only. To examine whether there was a reduction in breast cancer risk among the most active 5% of women in the cohort, we divided the highest moderate plus vigorous physical activity level into two finer levels. Again, we observed no association.

On the 1991 questionnaire, the same physical activity questions that appeared on the baseline 1989 survey were asked again. When we repeated the analyses described in the preceding paragraph using the average of 1989 and 1991 hours per week as the exposure of interest, again we found no association between physical activity and breast cancer risk; all patterns of RR closely resembled those reported in Table 4. Finally, we examined the joint pattern of physical activity from late adolescence and recent adulthood (1989). Compared with women who were in the lowest physical activity levels in both adolescence and recent adulthood (1989), women who were in the highest activity levels at both these times had a RR of 1.0 (95% CI = 0.6–1.5).

If physical activity influences breast cancer risk by modifying ovarian hormone levels, this effect may be obscured by the use of oral contraceptives. We hy-

### Table 2. Characteristics of participants according to average level of recent (1989) moderate plus vigorous physical activity* (h/wk)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>&lt;1</th>
<th>1–1.9</th>
<th>2–3.9</th>
<th>4–6.9</th>
<th>≥7</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of participants</td>
<td>35 573</td>
<td>18 005</td>
<td>22 233</td>
<td>13 570</td>
<td>15 087</td>
</tr>
<tr>
<td>Mean age (standard deviation)</td>
<td>34.7 (4.6)</td>
<td>34.4 (4.6)</td>
<td>34.3 (4.6)</td>
<td>34.2 (4.7)</td>
<td>33.8 (4.8)</td>
</tr>
<tr>
<td>Menarche ≥14 y of age (%)</td>
<td>17.1</td>
<td>17.8</td>
<td>17.9</td>
<td>18.0</td>
<td>19.4</td>
</tr>
<tr>
<td>Nulliparous (%)</td>
<td>24.5</td>
<td>27.6</td>
<td>31.4</td>
<td>35.9</td>
<td>41.3</td>
</tr>
<tr>
<td>Current oral contraceptive use (%)</td>
<td>11.0</td>
<td>12.6</td>
<td>13.4</td>
<td>13.4</td>
<td>16.1</td>
</tr>
<tr>
<td>Never oral contraceptive use (%)</td>
<td>17.2</td>
<td>16.3</td>
<td>16.1</td>
<td>16.4</td>
<td>16.9</td>
</tr>
<tr>
<td>History of benign breast disease (%)</td>
<td>28.6</td>
<td>28.8</td>
<td>28.9</td>
<td>28.6</td>
<td>28.0</td>
</tr>
<tr>
<td>History of breast cancer in mother and/or sister (%)</td>
<td>6.1</td>
<td>6.1</td>
<td>5.6</td>
<td>6.2</td>
<td>5.7</td>
</tr>
<tr>
<td>Never smokers (%)</td>
<td>74.7</td>
<td>73.0</td>
<td>73.4</td>
<td>72.7</td>
<td>72.6</td>
</tr>
<tr>
<td>No recent alcohol consumption (%)</td>
<td>31.0</td>
<td>29.6</td>
<td>26.6</td>
<td>24.3</td>
<td>23.7</td>
</tr>
<tr>
<td>Irregular menstrual cycles in high school (%)</td>
<td>28.8</td>
<td>28.5</td>
<td>28.6</td>
<td>29.6</td>
<td>30.3</td>
</tr>
<tr>
<td>Average menstrual cycle length ≥40 days between ages 18 and 22 (%)</td>
<td>7.8</td>
<td>7.8</td>
<td>7.5</td>
<td>7.7</td>
<td>7.8</td>
</tr>
<tr>
<td>Body mass index &gt;23.0 at age 18 y (%)</td>
<td>22.5</td>
<td>21.0</td>
<td>20.1</td>
<td>19.4</td>
<td>20.1</td>
</tr>
<tr>
<td>Body mass index &gt;25.0 in 1989 (%)</td>
<td>37.4</td>
<td>31.8</td>
<td>27.6</td>
<td>24.5</td>
<td>22.2</td>
</tr>
<tr>
<td>Weight gain &gt;10 kg, age 18 y to 1989 (%)</td>
<td>38.1</td>
<td>31.9</td>
<td>26.9</td>
<td>22.9</td>
<td>21.0</td>
</tr>
<tr>
<td>Height &gt;67 inches (%)</td>
<td>14.7</td>
<td>15.8</td>
<td>16.1</td>
<td>16.3</td>
<td>17.1</td>
</tr>
</tbody>
</table>

*Estimates adjusted for age at baseline, age at menarche, history of benign breast disease, history of breast cancer in mother and/or sister, recent alcohol consumption, height, oral contraceptive history, and parity and age at first birth (combination variable).

### Table 3. Average level of vigorous physical activity during high school and between ages 18 and 22 years and relative risks (RRs) of breast cancer (95% confidence intervals [CIs])

<table>
<thead>
<tr>
<th>Person-years of observation (N = 518,297)</th>
<th>No. of subjects with breast cancer (N = 372)</th>
<th>Age-adjusted RR (CI)</th>
<th>Multivariate-adjusted* RR (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Never</td>
<td>90,489</td>
<td>77</td>
<td>1.0</td>
</tr>
<tr>
<td>1–3 mo’y</td>
<td>109,639</td>
<td>70</td>
<td>0.9 (0.6–1.2)</td>
</tr>
<tr>
<td>4–6 mo’y</td>
<td>126,440</td>
<td>93</td>
<td>1.1 (0.8–1.4)</td>
</tr>
<tr>
<td>7–9 mo’y</td>
<td>112,962</td>
<td>78</td>
<td>1.1 (0.8–1.5)</td>
</tr>
<tr>
<td>10–12 mo’y</td>
<td>78,768</td>
<td>54</td>
<td>1.1 (0.8–1.6)</td>
</tr>
</tbody>
</table>
wider (data not shown).

from 1.0, although CIs were obviously activity measures were indistinguishable

(22) notes, however, in the epidemiologic studies that have shown that a reduced risk of breast cancer is associated with exercise, it is similarly unlikely that the most active women were engaging in competitive athletic training. She concluded that delayed onset and decreased numbers of ovulatory cycles cannot be the only explanation for the observed association between exercise and breast cancer. In postmenopausal women, physical activity has been hypothesized to reduce breast cancer risk in part by reducing fat stores, and thus endogenous estrogen levels. However, among premenopausal women in high-risk countries, higher levels of adiposity have been consistently associated with a reduced risk of breast cancer (23).

Although there are currently no proven mechanisms to explain this inverse association, it is plausible that physical activity conveys less protection in postmenopausal women than in premenopausal women and Bernstein et al. (9) showed a strong inverse association for a sample of women aged 40 years and younger.

It is possible that error in our measurement of physical activity levels could have attenuated a real protective effect of activity in these data. In particular, long-term recall of physical activity during late adolescence may be difficult. Although we cannot completely exclude this possibility, we have good evidence that our measures of activity were informative with regard to the specified time periods. The measures correlate as expected with age at menarche, BMI at age 18 years, current BMI, and weight gain. Although we obtained no validation data on the measure of late adolescent physical activity, in a validation study of the instrument used to assess recent activity (24), the Pearson correlation coefficient between physical activity reported in 7-day diaries (mailed out at intervals of 3 months over the course of a year) and the questions used on the 1989 survey were relatively high (.62) for a representative sample of women. This physical activity assessment instrument has been shown to be predictive of several disease outcomes, including noninsulin-dependent diabetes mellitus in women (25), colon cancer in men and women (26,27), and gallstones in men (28).

Currently, there is no consensus regarding the critical time period in which to measure physical activity in terms of its effect on breast cancer risk. Physical activity at a certain time in life may exert different effects on premenopausal versus postmenopausal breast cancer risk, because the associations between weight (and weight gain) and breast cancer risk may vary by menopausal status (23,29). Furthermore, if the critical exposure of interest is the lifelong physical activity pattern, as suggested by Bernstein et al. (9), then our assessment of physical activity level pertaining to only two limited time periods will result in misclassification in terms of the true exposure of interest. The women in our study showed considerable variability in physical activity over even short periods of time. For example, when we compared responses for high school and ages 18–22 years, less than half (44.6%) of the women in our study gave the same answer to the question, “How many months per year did you engage in strenuous physical activity at least two times per week”? Of those who answered “10–12 months” for the years spent in high school, only 30% gave this answer for the age period 18–22 years. Of those most physically active in late adolescence (according to the averaged high school and age 18–22-year measure), only 27% were in the highest level of moderate plus vigorous activity in 1989, and nearly 20% of those most physically active in late adolescence were in the least active category in 1989. Considering the short time interval of 1989 to 1991, only 39% of the women remained in the same moderate plus vigorous activity level (based on number of hours of physical activity/
week) on the two questionnaires. Of those most active in 1989, only 20% remained in the most active level in 1991, while 25% of those most active in 1989 had dropped to the least active group in 1991. Thus, no one of our measures could be taken as reflective of lifelong physical activity, and to crudely average them would ignore the sometimes substantial gap in time between ages 18 and 22 years and 1989. However, in most of the other studies (10,11,14,16,17) in which inverse associations were observed, the physical activity assessment method was not more detailed than ours and did not assess lifetime activity.

In summary, we found no support for the hypothesis that higher levels of physical activity in late adolescence or in recent adulthood reduces premenopausal breast cancer risk. The inconsistency in epidemiologic findings, however, demands a more detailed examination of physical activity throughout life in relation to both premenopausal and postmenopausal breast cancer. As noted by a recent review of this topic (30), future studies should focus on improving assessment of lifetime physical activity from all sources to clarify whether there is a dose–response relationship or an optimal time period, frequency, or intensity of physical activity with respect to reducing breast cancer risk.

References


Notes

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Availability of PSC833, a Substrate and Inhibitor of P-glycoproteins, in Various Concentrations of Serum

Alexander J. Smith, Ulrich Mayer, Alfred H. Schinkel, Piet Borst*

Background: P-glycoproteins are membrane-associated transporters that can render cells resistant to a variety of chemotherapeutic drugs. Reversal agents are (preferably nontoxic) drugs that can inhibit these P-glycoproteins and thereby overcome multidrug resistance. PSC833, a cyclosporin A analog, is a reversal agent that has shown potential in vitro experiments and in clinical trials. We tested PSC833 to determine whether it is a transported substrate of human and murine P-glycoproteins associated with multidrug resistance (encoded by the human MDR1 gene and its murine homolog, mdrla) and whether it can completely inhibit these P-glycoproteins under simulated in vitro conditions. Methods: Monolayers of polarized LLC-PK1 pig kidney cells transfected with complementary DNA containing either MDR1 or mdrla sequences were used to measure the directional transport of P-glycoprotein substrates under various serum conditions. Results: In contrast to two previous studies, we found that PSC833 is transported by both the MDR1 and the mdrla P-glycoproteins, albeit at a low rate. PSC833 has a very high affinity for the MDR1 P-glycoprotein, and its Michaelis constant ($K_m$) for transport is 50 nM, fourfold lower than for cyclosporin A. Inhibition of drug transport by PSC833 is approximately eightfold less effective in 100% fetal bovine serum than in tissue culture medium containing 10% serum. The concentration of PSC833 necessary to fully inhibit transport of digoxin and paclitaxel (Taxol) under complete (i.e., 100%) serum conditions is higher than the plasma concentrations achieved in clinical trials. Conclusions: Although PSC833 binds efficiently to the MDR1 P-glycoprotein and is released only sluggishly, the high concentrations of PSC833 necessary to inhibit this P-glycoprotein under complete serum conditions in our in vitro system suggest that it may be difficult for PSC833 alone to produce total inhibition of P-glycoprotein activity in patients. [J Natl Cancer Inst 1998;90:1161–6]

Mammalian cells can become resistant to several chemotherapeutic drugs after selection with a single drug in vivo or in vitro. This multidrug resistance can be caused by P-glycoproteins (P-gps) (1,2) encoded by the human multiple drug resistance 1 (MDR1) gene and the murine mdrla and mdrlb genes (also known as md3 and mdrl, respectively). P-gps are cell membrane proteins that render cells resistant to cytotoxic drugs by actively extruding drugs from the cytoplasm into the extracellular space, resulting in a decreased drug concentration inside the cell (3–6).

P-gp-mediated multidrug resistance can be overcome by coadministration of reversal agents that act as inhibitors of P-gps, such as verapamil and cyclosporin A (CSA) (7–9). Reversal agents are effective in vitro, but trials testing the inhibitory characteristics of these agents. Hence, the current interest in second-generation agents with more favorable side effects. One of the most effective of these is PSC833, a nonimmunosuppressive analog of CsA (13,14). PSC833 can completely inhibit human P-gp in vitro at concentrations that can be tolerated in vivo and is being tested in clinical trials (15,16).

In studies with wild-type mice and mice homozygous for a disruption of the mdrla and mdrlb genes (mdrla/b [−/−] mice) (17), we noted that the maximal concentrations of PSC833 tolerated failed to completely inhibit P-gp in the blood-brain barrier (18). In the same study, we found that PSC833 appears to be actively transported out of the brain in mice with functional P-gp. Analogous observations were made by Lemaire et al. (19) in rats. These results are in contrast with some in vitro studies that reported that PSC833 is not transported by P-gp (20,21).

To resolve the discrepancies between our work and previous studies, we analyzed the behavior of PSC833 in monolayers of polarized kidney cells transfected with P-gp genes (22). Transport of compounds through this monolayer can be measured with high sensitivity and the ability of PSC833 to inhibit drug transport can be tested under conditions more resembling the in vivo situation (i.e., in complete serum rather than the 10% serum normally used for studying reversal agents in vitro).

Materials and Methods

[12x-3H(N)]digoxin (0.40 GBq/mmol) and [3H]paclitaxel (0.12 GBq/mmol) were obtained from Du Pont NEN, Boston, MA; inulin [14C]carboxylic acid (molecular weight approximately 5200) was obtained from Amersham Life Science Inc., Arlington Heights, IL; [3H]paclitaxel (0.31 GBq/mmol) was obtained from Moravek Biochemicals, Inc., La Brea, CA; t-[1-14C]Val SDZ PSC833 (1.47 MBq/mmol) was from Sandoz Pharma Ltd. (now Novartis Pharma Inc.), Basel, Switzerland; SDZ PSC833 was from Sandoz Pharma Nederland B.V., Uden, The Netherlands; all tissue culture materials were from GIBCO BRL Paisley, Scotland; and other chemicals were from Sigma Chemical Co., St. Louis, MO.

Tissue Culture

The LLC-PK1 pig kidney epithelial cells (American Type Culture Collection, Manassas, VA) were cultured in M199 medium supplemented with 50 U of penicillin/mL, 50 μg of streptomycin/mL, and 10% fetal bovine serum (FBS) at 37 °C in 5% CO2. The cells were trypsinized and subcultured every 3–4 days. The MDR1- and mdrla-transfected clones of LLC-PK1 (22) were maintained routinely in the absence of drugs and tested for P-gp content on protein immunoblots regularly as described previously (22,23).

Transport Assays

Drug transport assays were generally performed as described previously (22,24). Cells were seeded on microporous polycarbonate membrane filters (pore size 3.0 μm, diameter 24.5 mm, Transwell™, Comina Costar Corp., Cambridge, MA) at a density of 1.5–2 × 105 cells per well. The cells were grown

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in complete medium, with a fresh medium replacement the day after seeding, and reached confluency after 3 days. The paracellular flow was monitored during transport experiments by the appearance of inulin [14C]carboxylic acid (0.025 μCi/mL, 4.2 μM) in the opposite compartment and was always less than 1.5% of total radioactivity per hour. One hour before the start of the experiment, medium on both sides of the monolayer was replaced. The experiment was started by replacing the medium on either the apical or the basolateral side by medium containing [14C]PSC833 (54.5 μCi/μmol). The cells were incubated at 37 °C in 5% CO2, and aliquots were taken from both compartments at 1, 2, 3, and 4 hours. The appearance of radioactivity in the opposite compartment was measured and presented as the fraction of total radioactivity added at the beginning of the experiment. Directional transport was measured in duplicate and the range is shown.

Inhibition of drug transport by PSC833 was measured similarly. One hour before the start of the experiment, the medium on both sides of the monolayer was replaced with complete medium (including 10% FBS) or with 100% FBS containing the appropriate concentration of PSC833. Digoxin, paclitaxel (Taxol; Sigma Chemical Co., St. Louis, MO), and PSC833 were added as solutions in 96% ethanol. When required, extra ethanol was added to exclude an effect of the solvent. At the start of the experiment, the serum or medium in one of the compartments was replaced by serum or medium with 2 μM [3H]digoxin, 2 μM [3H]daunorubicin, or 2 μM [3H]paclitaxel (0.25 μCi/mL) and the required amount of PSC833. Samples were taken as described above. These experiments could not be done with human blood plasma instead of FBS because the tight kidney cell monolayers started to leak as soon as the plasma was added.

The ability of the cells to accumulate drugs at one side of the monolayer was measured by adding 2 mL of medium containing equal concentrations of (radioactive) drug to both sides of the monolayer. At 1, 2, 3, and 4 hours, 50-μL samples were taken and the amount of drug in each compartment was determined. If the drug is transported actively by P-gp, the amount of drug in the apical compartment should increase as the amount of drug in the basolateral compartment decreases. A concentration gradient is thus formed and maintained.

**Statistics**

In the transport experiments, two replicate slopes were measured per experiment. Statistical analysis of the experiments was performed by a one-way analysis of variance approach with the individual slopes as experimental units. The slope of the line through the four time points of each well was determined, resulting in two independent estimations of the slope per experiment. Because we assumed that the random variation of the slope was equal in all experiments, we used the differences between the two independent estimations of all experiments to determine this random variation. All P values are two-sided. Differences are considered significant if P<.05.

**Calculations**

An estimation of transport kinetics of PSC833 was derived by making the following simplifying assumptions. 1) Active transport in the parent cell line is negligible as shown in Fig. 1. 2) The apical and basolateral membranes are comparable in surface and permeability. On expression of MDR1, the increase in the flux from basolateral to apical is approximately equal to the decrease in the flux from apical to basolateral, indicating that there is no large difference in the amount of drug that can diffuse through the two membranes over time. 3) Free PSC833 is homogeneously present inside the cell. Obviously, a significant part of the intracellular PSC833 will insert in intracellular membranes, but this drug will not be available for inhibition; our calculations only concern free PSC833, available for inhibition of P-gp. 4) Drug flow is linear over 4 hours. After a short initial accumulation period, the drug concentration inside the cell reaches a constant value. During this intracellular steady state, the following equation applies:

\[
\text{Influx} = (\text{efflux})_{\text{apically}} + (\text{efflux})_{\text{basolaterally}}
\]

\[
\text{Influx} = k[P] + (V_{\text{max}}[P]/(K_m + [P]))
\]

\[
\text{where} \quad k = k_{\text{p}} + k_{\text{i}} + k_{\text{B}} + k_{\text{j}}
\]

1) Active transport in the parent cell line is negligible as shown in Fig. 1. 2) The apical and basolateral membranes are comparable in surface and permeability. On expression of MDR1, the increase in the flux from basolateral to apical is approximately equal to the decrease in the flux from apical to basolateral, indicating that there is no large difference in the amount of drug that can diffuse through the two membranes over time. 3) Free PSC833 is homogeneously present inside the cell. Obviously, a significant part of the intracellular PSC833 will insert in intracellular membranes, but this drug will not be available for inhibition; our calculations only concern free PSC833, available for inhibition of P-gp. 4) Drug flow is linear over 4 hours. After a short initial accumulation period, the drug concentration inside the cell reaches a constant value. During this intracellular steady state, the following equation applies:

\[
\text{Influx} = (\text{efflux})_{\text{apically}} + (\text{efflux})_{\text{basolaterally}}
\]

\[
k[P]_{\text{apical}} = k_{\text{p}}[P]_{\text{apical}} + (V_{\text{max}}[P]/(K_m + [P]))
\]

\[
+k[P]_{\text{basolateral}}
\]

\[
\text{Influx} = k_{\text{p}}[P]_{\text{apical}} + k_{\text{i}}[P]_{\text{basolateral}} + (V_{\text{max}}[P]/(K_m + [P]))
\]

\[
\text{Influx} = k_{\text{p}}[P]_{\text{apical}} + k_{\text{i}}[P]_{\text{basolateral}} + (V_{\text{max}}[P]/(K_m + [P]))
\]

\[
k_{\text{p}}[P]_{\text{apical}} + k_{\text{i}}[P]_{\text{basolateral}} + (V_{\text{max}}[P]/(K_m + [P]))
\]

\[
\text{Influx} = k_{\text{p}}[P]_{\text{apical}} + k_{\text{i}}[P]_{\text{basolateral}} + (V_{\text{max}}[P]/(K_m + [P]))
\]

\[
\text{Influx} = k_{\text{p}}[P]_{\text{apical}} + k_{\text{i}}[P]_{\text{basolateral}} + (V_{\text{max}}[P]/(K_m + [P]))
\]

\[
\text{Influx} = k_{\text{p}}[P]_{\text{apical}} + k_{\text{i}}[P]_{\text{basolateral}} + (V_{\text{max}}[P]/(K_m + [P]))
\]

\[
\text{Influx} = k_{\text{p}}[P]_{\text{apical}} + k_{\text{i}}[P]_{\text{basolateral}} + (V_{\text{max}}[P]/(K_m + [P]))
\]

\[
\text{Influx} = k_{\text{p}}[P]_{\text{apical}} + k_{\text{i}}[P]_{\text{basolateral}} + (V_{\text{max}}[P]/(K_m + [P]))
\]

\[
\text{Influx} = k_{\text{p}}[P]_{\text{apical}} + k_{\text{i}}[P]_{\text{basolateral}} + (V_{\text{max}}[P]/(K_m + [P]))
\]

\[
\text{Influx} = k_{\text{p}}[P]_{\text{apical}} + k_{\text{i}}[P]_{\text{basolateral}} + (V_{\text{max}}[P]/(K_m + [P]))
\]
In the parent cell line LLC-PK1, active transport of 2 μM PSC833 is negligible and thus:

$$k[P_i] = (k[P_i])_b + (k[P_i])_a \rightarrow [P_i] = 2[P_i]$$

From the graph (Fig. 1; LLC-PK1, 2 μM PSC833) it can be deduced that $k[P_i] = 14.7 \times 2 \mu M \times 2 \mathrm{mL} \times (240 \mathrm{minutes})^{-1} \times \mathrm{well}^{-1} = 2.45 \times 10^{-12} \mathrm{mol/minute/well}.

$$k = 2.45 \times 10^{-6} \mathrm{L/minute/well}.$$

This passive permeation coefficient will also be valid for the transfected LLC-PK1 cell lines with this concentration of drug. At lower drug concentrations, this value tends to decrease, possibly because at these very low concentrations relatively more PSC833 may bind to the material of the wells.

$$(k[P_i])_b$$ is the flux to the basal compartment in the assay. The value of $(k[P_i])_b$ can be obtained from the data in Fig. 1 for LLC-1.1 cells; the value of $k$ is calculated from the data in Fig. 1 for LLC-PK1 cells as above and will not depend on the cell type. These two values can be used to calculate the drug concentration in the MDR1-transfected cells: $[P_i] = (k[P_i])_b/k$ (Table 1).

The active transport $[(V_{max}[P_i], k[K_m + P_i])]$ is the total flux to the apical compartment minus the passive diffusion to the apical compartment. Because the apical and basolateral membranes are assumed equal, the passive efflux to either side will be equal. Therefore, the active transport is the net difference between the flux from basolateral to apical and vice versa in a transport experiment.

### Results

**[14C]PSC833 Transport by P-gps**

Fig. 1 shows the PSC833 transport rate at three concentrations of [14C]PSC833 through monolayers of LLC-PK1-derived cell lines. At all concentrations, the fractional transport to the apical compartment is significantly higher in the MDR1-transfected cell line LLC-1.1 than in the parent cells, whereas the fractional transport in the opposite direction is correspondingly lower. Vectorial transport is due to the presence of MDR1 P-gp in the apical membrane of the transfected cells (22,24). Net transport of PSC833 through the monolayers of the mdr1a-transfected cell line LLC-1a is as high as through monolayers of the MDR1 transfectant, showing that the reversal agent is a substrate of both MDR1 and mdr1a P-gp.

To test whether PSC833 transport by P-gp can take place against a concentration gradient, we measured the distribution of PSC833 in the two compartments, with 0.05 μM PSC833 initially on both sides of the monolayer. After 8 hours, 65% of the PSC833 accumulated in the apical compartment and 30% was in the basolateral compartment, demonstrating that MDR1 P-gp can transport this substrate against a concentration gradient (results not shown).

Table 1 presents our calculated values for PSC833 transport kinetics in the MDR1 transfectant LLC-1.1 (see "Materials and Methods" for details). The estimated $K_m$ and $V_{max}$ values for transport of PSC833 by MDR1 in the LLC-1.1 cell line are 50 nM and 0.6 pmol per minute/well, respectively. A comparable analysis of CsA transport gave a $K_m$ of 200 nM and a $V_{max}$ of 11 pmol per minute/well.

The $K_m$ for transport of daunorubicin was reported to be 1–2 μM (25). Comparison of the reported values for the $V_{max}$ in different systems is not meaningful because these values are dependent on the transport assay system used.

### Inhibition of Drug Transport by PSC833 in Culture Medium and in Serum

A recent in vivo study (18) with mice suggested that the inhibition of mdr1a P-gp by PSC833 may be incomplete even at high plasma concentrations of the drug.

### Table 1. Calculated kinetic values of PSC833 transport through monolayers of MDR1-transfected LLC-1.1 cells after 4 hours*

<table>
<thead>
<tr>
<th>$[P_i]$ μmol/L</th>
<th>% per 4 h</th>
<th>pmol/min per well</th>
<th>$[P_i]$ μmol/L</th>
<th>% per 4 h</th>
<th>pmol/min per well</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max} \cdot [P_i]/K_m + [P_i]$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12.3</td>
<td>2.05</td>
<td>8.8 × 10^{-1}</td>
<td>3.9</td>
<td>0.633</td>
</tr>
<tr>
<td>0.4</td>
<td>6.05</td>
<td>0.202</td>
<td>1.1 × 10^{-1}</td>
<td>11.54</td>
<td>0.385</td>
</tr>
<tr>
<td>0.2</td>
<td>4.24</td>
<td>0.0707</td>
<td>3.6 × 10^{-2}</td>
<td>13.43</td>
<td>0.224</td>
</tr>
<tr>
<td>0.1</td>
<td>2.41</td>
<td>0.0201</td>
<td>1.0 × 10^{-2}</td>
<td>13.51</td>
<td>0.113</td>
</tr>
</tbody>
</table>

* $[P_i]$ is the PSC833 concentration in the medium; $(k[P_i])_b$ is the passive efflux of PSC833 to the basolateral compartment; $[P_i]$ is the intracellular PSC833 concentration; and $(V_{max}[P_i]/(K_m + [P_i])$ is the active transport of PSC833 to the apical compartment.
Because comparable concentrations of PSC833 can completely inhibit P-gp activity in cultured cells in tissue culture medium (26), it seemed possible that the differences between in vitro and in vivo results could be due to extensive binding of PSC833 to serum components. We therefore compared inhibition of drug transport by PSC833 in tissue culture medium (containing 10% FBS) and in 100% FBS. Fig. 2 shows that the transport rate of the substrate drug digoxin (22,24,27) through monolayers of LLC-1.1 cells is not significantly affected in 100% FBS compared with tissue culture medium. Addition of 1 µM PSC833, a concentration that gives a complete inhibition in tissue culture medium, does not significantly inhibit digoxin transport in 100% FBS, showing that PSC833 is less effective in 100% serum than in tissue culture medium. The inhibitory activity of PSC833 in serum-free medium, in normal medium (containing 10% FBS), and in 100% FBS on the transport of digoxin, daunorubicin, and paclitaxel is shown in Fig. 3. The concentrations of PSC833 that inhibit drug transport by 50% (i.e., its IC₅₀) in 100% serum are 3.0, 1.5, and 5.7 µM for digoxin, daunorubicin, and paclitaxel, respectively. These IC₅₀ values are eightfold to ninetofold higher than the values in normal tissue culture medium. In medium without any serum, the IC₅₀ value for digoxin transport is 23-fold lower than in 100% serum (Fig. 3, A).

Discussion

Our results show that both MDR1 P-gp and mdr1a P-gp can transport PSC833 against a concentration gradient, although the transport rate is low (Fig. 1). The MDR1 P-gp has a high affinity for PSC833, as would be expected for such a potent reversal agent. The Kₐ of MDR1 P-gp for CsA, the parent drug of PSC833, is approximately 200 nM in our system, fourfold higher than for PSC833. The maximal transport rate of PSC833 is 0.6 pmol per minute/well, whereas CsA can be transported at a rate almost 20 times higher.

The high affinity of PSC833 for P-gp and its low maximal transport rate are probably the reason why PSC833 is a better reversal agent than CsA. Both reversal agents bind to P-gp with high affinity, but the actual transport of PSC833 is much slower and thus PSC833 will occupy the P-gp molecule longer during transport. The rate of passive diffusion of CsA and PSC833 through the parent cell line LLC-PK1 is roughly the same (22). Because PSC833 enters the cell as efficiently as CsA but is transported out less effectively, in the presence of P-gp, the concentration of PSC833 inside the cell will also be higher than the concentration of CsA.

The low rate of PSC833 transport is probably the main reason why other studies (20,21) reported that MDR1 cannot transport this reversal agent. Only at low concentrations of PSC833 can active transport be measured against the background of the passive influx and efflux of the drug. Most transport studies with PSC833 have used much higher drug concentrations and a less sensitive readout. Hence, it is not surprising that no active transport of PSC833 was found.

Recent experiments with mice have suggested that PSC833 is a less effective inhibitor in blood than in tissue culture medium (18) as was also reported for several other reversal agents (28,29). We now find that the inhibitory activity of PSC833 is drastically reduced in 100% FBS relative to serum-free medium. The recent observation that 97%–99% of PSC833 in human blood plasma appears to be bound to serum lipoproteins (30) provides a plausible explanation for this decreased inhibitory activity. The high serum binding may at least partly explain the incomplete inhibition of P-gp by PSC833 in the mouse model. However, other factors (e.g., high nonspecific tissue binding) may also play a role.

In vitro, the influx of PSC833 into cells in monolayers may be less efficient than its influx into free-growing cells, for example, because the membrane surface area available is smaller. Permeation of drugs into solid tumors in vivo is often rather inefficient as well. The experiments with cells in monolayers may, therefore, mimic the in vivo situation better than experiments with free-growing cells. In vitro studies can never completely reproduce the true in vivo situation, however,
and this should be kept in mind in the following extrapolation of our in vitro results to patients.

Clinical trials with PSC833 have shown that the dose of PSC833 that can be administered to patients is limited by neurotoxicity, e.g., ataxia. The current intravenous administration regimen results in a mean blood concentration of approximately 2.2 μM (15,16). By oral administration of PSC833, a blood concentration of 1.6 μM is reached (31,32). Because PSC833 scarcely enters the erythrocytes, which constitute approximately half the volume of blood, the plasma concentration of PSC833 in these trials will be approximately twice as high. The PSC833 IC<sub>50</sub> values that we obtained in vitro for transport of digoxin, daunorubicin, and paclitaxel in serum are 3.0, 1.5, and 5.7 μM, respectively, suggesting that PSC833 may not be able to completely block P-gp activity in patients, especially the transport of paclitaxel.

The results that were obtained in the first phase I clinical trials show an increase in the area under the curve of the plasma concentration of PSC833 (15,16) due to the inhibition of P-gp activity in excretory organs. These results are in agreement with the results in wild-type and mdr1a/b (−/−) mice, obtained by Mayer et al. (18). In wild-type mice in those experiments, the P-gp in the animals’ excretory organs was completely inhibited by PSC833, whereas the P-gp in the tissues of the blood-brain barrier was inhibited only partially.

Even incomplete inhibition of P-gp by PSC833 might still be useful in overcoming multidrug resistance in patients. Moreover, combination of PSC833 with other reversal agents might result in complete P-gp inhibition, since the coadministration of several reversal agents has been shown to result in additive inhibitory effects on P-gp, whereas their toxicity is rarely additive (33,34). Nevertheless, our results indicate that PSC833 may not yet be the perfect reversal agent. Because the dose-limiting toxicity of PSC833 appears to be unrelated to its inhibition of P-gp, it might be possible to find analogs that bind even tighter to P-gp without an increase in toxicity.

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Notes

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