Response of Radiation-induced Fibrosarcoma-1 in Mice to Cyclophosphamide Monitored by *in Vivo* $^{31}$P Nuclear Magnetic Resonance Spectroscopy

Shi-Jiang Li, Janna P. Wehre, S. Sunder Rajan, R. Grant Steen, Jerry D. Glickson, and John Hilton

ABSTRACT

*In vivo* $^{31}$P nuclear magnetic resonance spectroscopy has been used to examine the RIF-1 fibrosarcoma in mice during untreated growth and following chemotherapy with cyclophosphamide. Levels of inorganic phosphate increase relative to phosphocreatine or nucleoside triphosphates during early untreated growth. After the tumor reaches a volume of approximately 1 g, no further decrease in energy level is observed. Following treatment with cyclophosphamide, tumor phosphorus metabolite ratios and pH are significantly altered, compared to untreated age-matched controls. During the growth delay period following chemotherapy there is a significant reduction in the ratio of inorganic phosphate to other phosphate metabolites, compared to age-matched controls. In addition, a more alkaline pH is observed in the tumors of treated animals. When the growth delay period ends, nuclear magnetic resonance spectra return to pretreatment patterns. The magnitude of the differences in $^{31}$P nuclear magnetic resonance spectral parameters between treated animals and untreated controls is dose dependent. However, doses of cyclophosphamide above 200 mg/kg do not result in earlier spectroscopic alterations, nor in larger effects by Day 3 after treatment, even though clonogenic cell killing and growth delay are greater at these higher doses.

INTRODUCTION

Using $^{31}$P NMR spectroscopy it is possible to make noninvasive *in vivo* measurements of several important phosphorus metabolites including ATP (together with other NTPs), PCr, and P$_i$, as well as average tissue pH. In studies of malignant tumors both in laboratory animals (1-4), and in humans (5-7) changes have been observed in $^{31}$P NMR spectra during untreated growth or after administration of an anticancer therapy. In many cases, administration of chemotherapy or X-irradiation is followed by a reversal of the trends observed during untreated growth (1-2). In contrast, following hyperthermia (8), certain changes have been observed in 31P NMR spectra of a variety of tissues. In preliminary experiments control and cyclophosphamide-treated tumors were examined with repetition delays of 3, 5, and 10 s. The small differences in the degree of partial saturation between treated and control tumors were negligible compared to the magnitude of the differences between treated and control tumors.

Neutralized perchloric acid extracts were prepared from freeze-clamped tumors of treated and control animals on the third day after treatment with 200 mg/kg of cyclophosphamide. *In vivo* spectra (not shown) were collected from each animal to ensure that the spectra were typical of those shown in Table 1, then three tumors were pooled for each extract, to provide sufficient material and to average the small variations. $^{31}$P NMR spectra were obtained at 8.5 T using a commercial 360 spectrometer (89-mm bore) at 8.5 T (145.8 MHz for phosphorus). The home-built probes contained two- or three-turn solenoidal radiofrequency coils (14), 1-2 cm in diameter. Copper Faraday shields around the body of the mouse were used to prevent accumulation of signals from outside the tumors (15). Coils and Faraday shields were size-matched to the tumors, to surround them closely without constriction. Each coil was tuned to 1 H for shimming and to $^{31}$P for data accumulation and carefully characterized with regard to 90° pulse lengths. Mice were anesthetized with sodium pentobarbital (65 mg/kg, i.p.), then allowed to rest with gentle warming for approximately 30 min prior to spectroscopic examination, which has been shown to allow full recovery of mouse blood pressure (17) and the $^{31}$P NMR spectrum (18). Each spectrum was obtained by accumulating 200 scans, using a 60° flip angle, and 1 K data points. A repetition time of 3 s was employed, which results in only a small partial saturation of PME and P$_i$. In preliminary experiments control and cyclophosphamide-treated tumors were examined with repetition delays of 3, 5, and 10 s. The small differences in the degree of partial saturation between treated and control tumors were negligible compared to the magnitude of the differences between treated and control tumors.

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RESPONSE OF RIF-1 TO CYCLOPHOSPHAMIDE

Untreated Tumor Progression. Fig. 1, left, gives an example of the 31P NMR spectra obtained from an individual RIF-1 tumor during untreated growth between Days 12 and 22 after inoculation. However, the spectra obtained on Day 12 (and following) were highly variable, even in size- and age-matched tumors. This has been noted previously by Evelhoch et al. (22) for larger RIF-1 tumors. In order to evaluate the significance of these changes during untreated growth a group of 15 tumor-bearing animals was studied. Fig. 2 illustrates the pattern of growth and metabolic change in this group. Changes in several 31P NMR parameters are correlated with growth during the second week of untreated growth. A large increase in Pj/βNTP and a smaller increase in Pj/PMÈ are observed during Days 10 to 15 postimplantation, while the mean tumor volume increases from 0.2 to 1.2 cm3. A significant decrease in PCr/Pj occurs during this time, indicating a decline in the average energy level of the tumor. After Day 15 tumor volume continues to increase, but no further change is observed in the mean values for the metabolite ratios. The mean tumor pH (not shown) drops from 7.27 (±0.10) on Day 10 to 7.07 (±0.12) on Day 13, after which no further significant decrease is observed.

Response to Cyclophosphamide. Fig. 1, right, illustrates the response of one RIF-1 tumor to treatment with cyclophosphamide. Before treatment this tumor has a higher Pj resonance (relative to other phosphorus resonances) than the tumor shown in Fig. 1, left, but it is within the range commonly observed for tumors of this age and size. Twenty-four h after treatment the Pj resonance is substantially smaller. An alkaline shift in the Pj resonance begins as a shoulder on the high field side of the Pj resonance (treated spectrum b). This alkaline component of the Pj resonance continues to grow until Day 5. When regrowth begins the pH returns to its original more acidic value and Pj rebounds.

Table 1 contains average metabolic ratios and tumor pH for a large group of tumors treated with cyclophosphamide (200 mg/kg). Because the spectroscopic properties of untreated tumors do not remain constant during the period under study, treated tumors were compared with age-matched, untreated controls, and not with pretreatment values. Twenty-four h after administration of this dose, Pj levels (relative to βNTP or PMÈ) are 25% lower than in control tumors. The PCr/Pj ratio is nearly double, but the pH is the same as untreated tumors. By the third day after treatment the differences in metabolite levels between treated and control tumors are even larger. This is due both to the progressive decrease in high energy phosphates in the untreated tumors, and to further improvement in the apparent energy status of the treated tumors. On the third day after treatment the PCr/Pj level of the treated tumors is 6-fold higher than that of age-matched controls. In addition, the average tumor pH has increased to 7.3, nearly 0.4 pH units more alkaline than control tumors. Six days following treatment these differences remain.

To examine the possibility that the apparent loss of Pj following treatment resulted from an increase in the fraction of Pj bound to macromolecules or sequestered in intracellular organelles, we examined the 31P NMR spectra of neutralized perchloric acid extracts of freeze-clamped tumors. Fig. 3 compares the extract spectrum of tumors 3 days after 200 mg/kg of cyclophosphamide with the spectrum of age-matched untreated tumors. The spectra are normalized to the height of the PMÈ resonance. The intensity of the Pj resonance relative to PMÈ, or relative to any of the other components, is clearly reduced in the treated tumors. Relative heights of other components are not substantially altered, suggesting that a difference in the degree of hydrolysis of intracellular PCr or ATP is not responsible for the loss of Pj.

The effect of cyclophosphamide (200 mg/kg) on the 31P NMR spectra of older, larger tumors (Table 1) is qualitatively similar to its effect on smaller tumors. These tumors were treated (or sham-treated) on Day 18 after inoculation, at an average volume of 2.2 cm3, 10 times larger than the tumors treated on Day 12. By the third day following treatment a decrease in Pj/PMÈ and Pj/βNTP ratios can be clearly seen, as well as an alkaline shift in tumor pH compared to age-matched controls. On the sixth day after late-stage treatment, the average PCr/Pj ratio in the treated tumors is nearly eight times greater than in 24-day-old untreated tumors.

When both treated and age-matched control tumors are examined, a correlation can be seen between average tumor pH and the value for PCr/Pj (Fig. 4). The data for all animals (treated plus control) can be fit to an empirical logarithmic relationship between pH and PCr/Pj, with a coefficient of determination R2 = 0.733 + 4.14 (log PCr/Pj) with a correlation coefficient r = 0.8 (P < 0.0005). However, neither group alone suggests such a relationship. It is clear from Fig. 4 that a combination of pH and PCr/Pj can distinguish treated from control tumors with a very high degree of accuracy on the third day following treatment. Linear discriminant analysis was used to generate the straight lines that best divide treatment and control groups on the third day after treatment, using various combinations of 31P NMR parameters (data from Table 1). A combination of pH and PCr/Pj values can correctly classify 100% of the observations as treated or control. This can be seen graphically in Fig. 4, where any one of several straight lines relating pH and PCr/Pj can discriminate between every treated and control tumor, although neither variable alone gives perfect prediction. A combination of the values for pH and Pj/βNTP can also give 100% correct classification, but the best line combining values for pH and PMÈ/βNTP can give no better than 92% correct classifications.
RESPONSE OF RIF-1 TO CYCLOPHOSPHAMIDE

Fig. 1. Typical in vivo $^3$P NMR spectra of s.c. RIF-1 tumors during growth, collected as described in "Materials and Methods." Resonance assignments are indicated. NAD(P)(H) indicates total pyridine nucleotides. Left spectra, from a single sham-treated tumor. Right spectra, from a tumor treated with 150 mg/kg of cyclophosphamide. Treatment or sham-treatment was performed on Day 12 after inoculation. A, Day 0 (before treatment or sham treatment); B, 1 day after treatment; C, 2 days; D, 3 days; E, 5 days; F, 7 days. PDE, phosphodiester compounds.

0.001). The difference between treated and control is better accounted for the model which uses pH and PCr/Pi values than by a model which only contains pH and Pi/βNTP ($R^2 = 0.740$). The values for PME/βNTP, in contrast, account for relatively little of the variation between treated and control tumors ($R^2 = 0.342$), and the fit of the regression is not improved significantly ($P = 0.52$) when this parameter is added to "pH plus PCr/Pi."

Dose Response of RIF-1 to Cyclophosphamide. The dose-response curve for values of NMR spectral parameters on Day 3 after cyclophosphamide administration was compared with the dose-response curves of two traditional therapeutic endpoints under identical conditions of tumor growth and treatment. Tumor volume changes following cyclophosphamide treatment demonstrate increasing therapeutic efficacy with increasing dose over the range 100–300 mg/kg (Fig. 5). In vivo-in vitro clonogenic cell survival assay (Fig. 6) indicates a logarithmic decrease in clonogenic cell survival with increased drug dose. Fig. 7 shows the values of several $^3$P NMR parameters on the third day after treatment with these doses of cyclophosphamide. At all doses the results are qualitatively similar. Significantly greater effects are observed with 200 mg/kg than with 100 mg/kg, for each NMR parameter shown ($P < 0.001$ for all except pH, for which $P < 0.025$). For some parameters (pH, Pi/PME) the maximum effect is observed at 150 mg/kg. For PCr/Pi and Pi/βNTP significantly larger effects are observed with 200 mg/kg than with 150 mg/kg ($P < 0.005$, $P < 0.01$, respectively). On Day 3 values for NMR spectral parameters after 300 mg/kg are not significantly different from those after 200 mg/kg ($P > 0.05$).

DISCUSSION

Untreated Growth. The relatively stable energy levels in large RIF-1 tumors may be contrasted with several other tumor lines.
such as the MOPC 104E myeloma (1) or the 9L gliosarcoma (23), in which the high energy phosphates virtually disappear as the tumor grows. Our study of RIF-1 indicates that until the tumor volume reaches 1–1.2 cm³ there is a progressive decrease in high-energy relative to low-energy phosphorus compounds during growth (Fig. 2). Beyond this point there is no further significant decrease in the sample mean energy status. There is, however, a substantial increase in variability, leading to observations similar to those of Evelhoch et al. (22), in which the levels of Pᵢ relative to high energy phosphates differed substantially in tumors of the same size and age. Although RIF-1 has been reported to have a low hypoxic fraction (1–1.5%, see References 12 and 24), those studies were performed on tumors with volumes of 200–400 mg, and the level of radiobiological hypoxia in larger tumors has not been determined.

Braunschweiger and Schiffer (25) found that for RIF-1 tumors above 1 g there was no change in the vascular volume or in total red blood cell volume, compared to smaller tumors. The tumor blood flow per gram (as a percentage of cardiac output) decreased linearly with increasing log tumor volume over the range 0.1–4.0 g. The size of the rapidly exchangeable fraction of the red blood cell pool in RIF-1 has been reported to be relatively constant during early growth, with a significant decrease only when tumors exceeded 1 g (25). The size of the well-perfused fraction measured by ¹⁵O washout was found to vary with tumor size over the entire range 0.2–2.0 g (22). Our results would be consistent with a decline of high energy phosphates during early growth due to the gradual reduction in tumor blood flow, with the low steady state level in individual larger tumors reflecting a residual well-perfused fraction measured by exchangeable red blood cell fraction (25).

Response to Chemotherapy. Treatment of RIF-1 with cyclophosphamide results in a rapid change in the relative levels of phosphorus metabolites. Significant differences between treated and control tumors in the level of Pᵢ relative to other resonances can be observed 24 h after a dose of 200 mg/kg or more. Differences are significant on the third day following treatment at all doses examined (100, 150, 200, and 300 mg/kg), although no significant tumor regression is observed during this time. In contrast to our previous experience (18), no animal deaths or obvious cyclophosphamide toxicity were observed in the present study. No spectra showed the very low NTP levels and extremely elevated Pᵢ resonances which we had previously observed in animals which subsequently died of apparent cyclophosphamide toxicity. For those earlier experiments RIF-1 tumors were induced in C3H/HeJ mice (The Jackson Laboratories). It is not known whether the difference in mouse strain was responsible for the increased drug toxicity.

The mechanism of the reduction in the Pᵢ resonance relative to other spectral resonances remains to be determined, but appears to involve, at least in part, a net loss of Pᵢ from the tumor. Changes in compartmentalization or binding of Pᵢ are not entirely responsible for the reduction in relative Pᵢ signal intensity, because spectra of tumor extracts also show a reduction in Pᵢ relative to other metabolites in treated compared to untreated tumors (Fig. 3). It does not appear that the Pᵢ has been reincorporated into NTP or PCr, however incorporation into acid-insoluble macromolecules or membrane-bound phospholipids cannot be ruled out. Incorporation into DNA is probably not responsible, as Pᵢ reduction occurs during the period when DNA synthesis is reduced following cyclophosphamide treatment (26, 27) will result in an apparent increase in Pᵢ relative to other spectral resonances remains to be determined, but appears to involve, at least in part, a net loss of Pᵢ from the tumor. Changes in compartmentalization or binding of Pᵢ are not entirely responsible for the reduction in relative Pᵢ signal intensity, because spectra of tumor extracts also show a reduction in Pᵢ relative to other metabolites in treated compared to untreated tumors (Fig. 3). It does not appear that the Pᵢ has been reincorporated into NTP or PCr, however incorporation into acid-insoluble macromolecules or membrane-bound phospholipids cannot be ruled out. Incorporation into DNA is probably not responsible, as Pᵢ reduction occurs during the period when DNA synthesis is reduced following cyclophosphamide treatment (26, 27) will result in an apparent increase in Pᵢ relative to other metabolites present only in the intracellular space. Thus, the actual decrease in the concentration of free Pᵢ,

### Table 1: Effects of cyclophosphamide on RIF-1 tumors

<table>
<thead>
<tr>
<th>Tumor age</th>
<th>Days post-treatment</th>
<th>Pᵢ/PME</th>
<th>Pᵢ/β-NTP</th>
<th>PCr/Pᵢ</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=15)</td>
<td>13 d</td>
<td>1 d</td>
<td>1.35 ± 0.37</td>
<td>1.69 ± 0.52</td>
<td>0.26 ± 0.11</td>
</tr>
<tr>
<td>Treated (n=17)</td>
<td>13 d</td>
<td>1 d</td>
<td>1.01 ± 0.36</td>
<td>1.29 ± 0.58</td>
<td>0.47 ± 0.23</td>
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<tr>
<td>Control (n=15)</td>
<td>15 d</td>
<td>3 d</td>
<td>1.57 ± 0.27</td>
<td>2.88 ± 1.31</td>
<td>0.16 ± 0.11</td>
</tr>
<tr>
<td>Treated (n=21)</td>
<td>15 d</td>
<td>3 d</td>
<td>0.60 ± 0.14</td>
<td>0.62 ± 0.17</td>
<td>0.96 ± 0.33</td>
</tr>
<tr>
<td>Control (n=15)</td>
<td>18 d</td>
<td>6 d</td>
<td>1.56 ± 0.62</td>
<td>2.91 ± 2.20</td>
<td>0.15 ± 0.11</td>
</tr>
<tr>
<td>Treated (n=8)</td>
<td>18 d</td>
<td>6 d</td>
<td>0.68 ± 0.11</td>
<td>0.61 ± 0.16</td>
<td>0.98 ± 0.51</td>
</tr>
<tr>
<td>Control (n=15)</td>
<td>21 d</td>
<td>3 d</td>
<td>1.44 ± 0.25</td>
<td>2.26 ± 0.83</td>
<td>0.14 ± 0.09</td>
</tr>
<tr>
<td>Treated (n=8)</td>
<td>21 d</td>
<td>3 d</td>
<td>0.70 ± 0.11</td>
<td>0.81 ± 0.12</td>
<td>0.33 ± 0.09</td>
</tr>
<tr>
<td>Control (n=15)</td>
<td>24 d</td>
<td>6 d</td>
<td>1.57 ± 0.37</td>
<td>2.94 ± 1.51</td>
<td>0.07 ± 0.04</td>
</tr>
<tr>
<td>Treated (n=8)</td>
<td>24 d</td>
<td>6 d</td>
<td>0.82 ± 0.13</td>
<td>0.74 ± 0.25</td>
<td>0.59 ± 0.22</td>
</tr>
</tbody>
</table>

* Significant differences: *, P < 0.025; **, P < 0.005; ***, P < 0.0005.
RESPONSE OF RIF-1 TO CYCLOPHOSPHAMIDE

Fig. 3. $^{31}$P NMR spectra of neutralized perchloric acid extracts of treated (3 days after 200 mg/kg) and age-matched control tumors. Tumors from three animals were combined for each spectrum. The line widths are broad because the samples were not treated to remove divalent cations.

Fig. 4. The relationship between tumor pH and PCr/P$_i$ ratio. Values for individual treated (200 mg/kg) or control tumors on the third day after treatment or sham-treatment. •, control tumors; O, treated tumors.

The data are consistent with the reduction in size of a compartment with a relatively high P$_i$ concentration. The tumor during the days after treatment is a mixture of dead and dying cells, undamaged cells and their progeny, and cells which have experienced genetic damage and are doomed to die (but may survive several days). The proportions of cells in these categories will be changing during the time after treatment. Preferential killing and loss of poorly energized cells with high intracellular P$_i$ concentration would be consistent with the apparent energy enhancement observed following cyclophosphamide treatment. On the other hand, areas of poorly perfused, hypoxic cells might be expected to be areas of relatively poor drug delivery. It may well be that although cells near blood
vessels are preferentially killed because of good drug delivery, the reduction in competition for available oxygen results in a high energy level for surviving cells, and that this is reflected in the in vivo spectrum of the total tumor. The radiobiological reoxygenation that follows tumor cell killing by X-irradiation has been attributed to a reduction in oxygen consumption relative to delivery (28). Increased oxygen delivery due to improved perfusion would also enhance the energy status of the tumor, however the changes in \( P_i \) observed following 150 mg/kg of cyclophosphamide precede the increases in blood flow measured following this dose (26).

Tumor \( \text{pH} \) is not significantly higher in treated animals than controls until the third day after treatment, even though \( \text{PCr}/P_i \) is significantly elevated on the first day. In earlier studies (11) the alkaline \( \text{pH} \) shift following treatment of RIF-1 with cyclophosphamide was also found to lag behind changes in the relative levels of phosphorus metabolites, and to occur simultaneously with an increase in tumor blood flow (26) and the recovery of cell proliferation (11). In a variety of normal and malignant cells, an alkaline shift in intracellular \( \text{pH} \) has been found to be correlated with the initiation of cell proliferation, stimulated by fertilization (29), by addition of serum or growth factors (30-33), or by treatment with specific mitogens (34). Decreased lactate production due to improved oxygenation, as well as increased lactate washout, would contribute to a decrease in tumor \( \text{pH} \) from increased blood flow. Like the value for \( P_i \), tumor \( \text{pH} \) will have an increasing contribution from the extracellular compartment during the days following cyclophosphamide treatment (26, 27). On the other hand, direct microelectrode measurements (35) have indicated that necrotic regions of tumors may have higher than average \( \text{pH} \) values. Treatment of the 9L gliosarcoma with 1,3-bis(2-chloroethyl)-1-nitrosourea, which causes an even more dramatic bioenergetic reactivation, is associated with a decrease in the incidence of necrosis (23). Histological analysis of RIF-1 will allow evaluation of this possible mechanism for the alkaline \( \text{pH} \) shift.

The apparent increase in tumor energy level following treatment is not restricted to small, relatively healthy tumors. Treatment of tumors 10 times larger than those usually studied results in levels of phosphorus metabolites and tumor \( \text{pH} \) similar to those observed after treatment of small tumors (Table 1). The large treated tumors do not achieve \( \text{PCr}/P_i \) values as high as smaller treated tumors, but the difference between treated and age-matched untreated tumors is even greater, because the energy status of the untreated tumors has declined even further. At a cyclophosphamide dose of 200 mg/kg, the response of the larger tumors is slower than the smaller tumors, both for \( \text{PCr}/P_i \) and for \( \text{pH} \), which are still increasing on Day 6 relative to Day 3. Smaller tumors exhibit their maximum response already by Day 3 posttreatment.

NMR-determined values for \( \text{pH} \) and \( \text{PCr}/P_i \) were found to be excellent discriminators between treated and untreated animals as early as the third day after chemotherapy. A combination of either \( \text{pH} \) plus \( P_i/\beta\text{NTP} \) or \( \text{pH} \) plus \( \text{PCr}/P_i \) results in 100% correctly classified results for treated versus control animals by linear discriminatory analysis. Either \( \text{pH} \) or \( \text{PCr}/P_i \) alone can account for 70% of the difference between treated and control tumors using binary regression analysis. The statistically significant improvement in predictive power of a model which includes both \( \text{pH} \) and \( \text{PCr}/P_i \) values suggests that these values do not identify an identical subset of tumor cells. Although RIF-1 does contain the enzyme creatine kinase, the heterogeneity of the tumor precludes any simple relationship between \( \text{pH} \) and metabolite levels. The cells, or even the regions of tumor which contain the \( \text{PCr} \) seen in in vivo spectra may be different from those which contain the high \( P_i \). In addition, the increased intracellular space represents a compartment in which the \( P_i \) and \( \text{pH} \) are not in equilibrium via creatine kinase with intracellular metabolites or \( \text{pH} \).

Clonogenic cell survival is a parameter closely related to the ultimate recurrence of the tumor, although it does not indicate how many cells die within the period of NMR observation. The parameters \( \text{PCr}/P_i \) and \( P_i/\beta\text{NTP} \) can distinguish the effects of different doses of cyclophosphamide from 100 to 200 mg/kg (Fig. 7). Clonogenic cell survival after 0, 100, 150, and 200 mg/kg is approximately 1, 0.05, 0.01, and 0.002, respectively (Fig. 6 and Reference 18). Further reduction in surviving fraction to approximately 0.0001 (300 mg/kg) caused no further change in the metabolite ratios by the third day following treatment. It is possible that at high doses maximum effects occur at a later date, however after the first few days comparisons between doses are complicated by the fact that tumors treated with low drug doses will escape from growth delay. When this happens, the NMR parameters return to control values and no longer reflect treatment. In contrast to the phosphate metabolites, the maximum change in \( \text{pH} \) was observed at the lowest dose given.

Conclusions. The fact that tumors appear better energized following treatments which result in massive cell killing seems paradoxical. It appears that in the case of cyclophosphamide, at least at the doses examined, posttreatment tumors are dominated by changes in tissue fluid spaces and perfusion, and by the effects of these changes on the cells which have not yet died. These results suggest that \( ^3\text{P} \) NMR spectroscopy will be a more sensitive indicator of therapeutic efficacy than might have been predicted.

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