**In vivo Anti-Cancer Activity of a Liposomal Nanoparticle Construct of Multifunctional Tyrosine Kinase Inhibitor 4-(4’-Hydroxyphenyl)-Amino-6,7-Dimethoxyquinazoline**

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**Abstract**

The quinazoline derivative 4-(4’-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P131/JANEX-1; CAS 202475-60-3) is a dual-function inhibitor of Janus kinase 3 (JAK3) and Epidermal Growth Factor (EGF) receptor kinase. A PEGylated liposomal nanoparticle formulation of GMP-grade WHI-P131 exhibited potent in vivo activity against breast cancer cells. Notably, this therapeutic nanoparticle formulation of GMP-grade WHI-P131 was substantially more effective than the standard chemotherapy drugs paclitaxel, gemcitabine, and gefitinib against chemotherapy-resistant breast cancer in the MMTV/Neu transgenic mouse model. These experimental results demonstrate that the nanotechnology-enabled delivery of WHI-P131 shows therapeutic potential against breast cancer.

**Keywords:** CAS 202475-60-3; JAK3; Quinazoline; GMP; WHI-P131; Breast cancer

**Introduction**

WHI-P131 is a dual-function inhibitor of JAK3 and EGF receptor tyrosine kinases [20]. It is being developed as a potential anti-cancer and immunomodulatory drug candidate [28,26]. WHI-P131 demonstrated potent in vivo anti-inflammatory and immunomodulatory activity in several preclinical animal models [3-7,13,14,26]. It has been shown that WHI-P131 exhibits potent pro-apoptotic anti-cancer activity against human cancer cells with constitutive JAK3/STAT3 activation [1,2,11,12,15,16,19,20] and displays chemopreventive properties in animal models of gastrointestinal neoplasia [25] and non-melanoma skin cancer [21]. WHI-P131 exhibited a favorable pharmacokinetics and safety profile in preclinical studies in rodents and monkeys [24]. Forty-eight distinct therapeutic liposomal nanoparticle constructs of WHI-P131 have been prepared and a PEGylated lead formulation (viz.: WHI-P131 [NP]) showed significant in vitro cytotoxicity against primary human leukemia cells from B-lineage acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) patients as well as potent in vivo anti-leukemic activity in a SCID mouse xenograft model of highly aggressive and radiochemotherapy resistant ALL [23]. WHI-P131 [NP] was substantially more potent in vivo than non-encapsulated WHI-P131 and drug-free nanoparticles exhibited no anti-cancer activity in the SCID mouse xenograft model [23]. The purpose of the present study was to further evaluate the therapeutic potential of WHI-P131 [NP] against chemotherapy-resistant breast cancer in the MMTV/Neu transgenic mouse model of metastatic ErbB2/HER2 breast cancer. In MMTV/Neu transgenic mice, the expression of wild-type rat Her2/neu gene is forced in the mammary gland under the control of the MMTV long terminal repeat. Neu transgenic mice develop rapidly progressive and metastatic breast cancer [22,27]. WHI-P131 [NP] was substantially more potent than the standard chemotherapy drugs paclitaxel, gemcitabine, and gefitinib at clinically applicable or higher dose levels and resulted in shrinkage of both primary and metastatic tumors in MMTV/Neu transgenic mice. These experimental results demonstrate that the nanotechnology-enabled delivery of WHI-P131 shows therapeutic potential against breast cancer.

**Materials and Methods**

**Preparation of WHI-P131 [NP]**

A PEGylated liposomal nanoparticle (NP) formulation of GMP-grade WHI-P131 (Encapsulated WHI-P131 concentration: 30.1±0.8 mg/mL; Approximate particle size after extrusion: 100 nm) was prepared using lipid film hydration, as described [23]. The liposome bilayer membranes of the nanoparticles were composed of dipalmitoylphosphatidylcholine (DPPC) and cholesterol [23]. Polyethylene glycol (PEG)-derivatized lipid 1,2-distearyl-sn-glycerol-3-phosphoethanolamine-n-poly(ethylene glycol) 2000 (DSPE-PEG2000) was also incorporated into the membranes for the purpose of enhanced steric stabilization [23].

**Animals**

We used the well established transgenic mouse model of ErbB2/HER2 breast cancer [22,27]. MMTV/Neu mice [FVB/N-TgN (MMTV neu) 202MUL; Jackson Laboratory, Bar Harbor, Maine] [22,27] were bred to produce multiple litters. All mice were housed in microisolator cages (Lab Products, Inc., Maywood, NY, USA) containing autoclaved bedding in a controlled specific pathogen-free (SPF) environment (12-h light/12-h dark photoperiod, 22±1°C, 60±10% relative humidity), which is fully accredited by the USDA (United States Department of Agriculture). Animal studies were approved by Parker Hughes Institute Animal Care and Use Committee and all animal care procedures conformed to the “Guide for the Care and Use of Laboratory Animals” published by the National Academy of Sciences (US National Academy of Sciences, 1996). All efforts were made to minimize suffering. All experiments were performed according to the guidelines set by the Institutional Animal Care and Use Committee (IACUC) and were approved by the IACUC of Parker Hughes Institute.

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Treatment of MMTV/Neu mice

Animals carrying one or more tumors were randomly placed in the study. Tumor-bearing mice were randomly assigned to PBS, WHI-P131-free vehicle, NP formulation of GMP-grade WHI-P131, paclitaxel (Taxol), gemcitabine (Gemzar) or gefitinib (Iressa) treatment groups. Chemotherapeutic drugs were obtained from the Parker Hughes Cancer Center Pharmacy (50 mg/kg, N=9; 100 mg/kg, N=9; 150 mg/kg, N=8). WHI-P131 [NP] (50 mg/kg, N=9; 100 mg/kg, N=9; 150 mg/kg, N=8) was administered by daily intraperitoneal injections on 5 consecutive days per week. Paclitaxel/Taxol (N=27) was administered intraperitoneally on days 1, 3, and 5 of each week at a dose level of 6.7 mg/kg. Gemcitabine (N=34) was administered on days 1 and 8 at a dose level of 33.7 mg/kg. Gefitinib (N=20) was suspended in distilled water and administered at 75 mg/kg dose in 0.2 ml by gastric gavage with a 20-gauge gavage needle. Control group (N=38) included mice that were treated daily for 5 days/week with ip injections of WHI-P131-free vehicle (n=9), WHI-P131 [NP] at the suboptimal 50 mg/kg dose level (N=9) or PBS (N=20). Tumor growth was determined by the measurement of tumors with a caliper in three dimensions three days a week and expressed as tumor volume in cubic millimeters (mm3). Tumor volumes were calculated using the formula for the volume of a prolate spheroid, V=4/3 x 3.14 x length/2 x width/2 x depth/2. Tumor size for each tumor was normalized to the starting volume of a prolate spheroid, V=4/3 x 3.14 x length/2 x width/2 x depth/2. Tumor size for each tumor was normalized to the starting volume for that particular tumor.

Statistical analysis

Tumor volume measurements were taken at day 1, 7 and 14 for control mice and those treated with WHI-P131 [NP], Gefinitib, Gemcitabine and Taxol. To investigate the treatment effect on the growth of tumors across 7 and 14 days we used an ANOVA model that accounted for variance components between mice and between initial tumor volumes at day 1. To control for mouse to mouse differences a random effect was included in the model using the REML method (Restricted or residual maximum Likelihood) for determining the variance component of this effect. Considerable variation was observed in tumor volumes at day 1 of the experiment, therefore, to assess the effect of tumor volume at day 1 and subsequent growth of tumors at days 7 and 14, the day 1 volume was included as a co-variate for the ANOVA models performed at days 7 and 14. A second interaction co-variate in the model controlled for differences in tumor volumes that were dependent on treatment (Day1*treatment interaction). These three control factors enabled testing of differences in tumor growth that accounted for mouse differences, multiple measurements taken from a mouse and tumor volume differences to follow growth over 14 days. We examined the distribution of the residuals of the model for equal dispersion around the line of best fit. We normalized all tumor volumes to day 1 measurements for control mice and those treated with WHI-P131 [NP], Gefinitib, gemcitabine, or gefitinib at the applied dose levels and treatment schedules (p<0.0001 for all comparisons), as documented by the significantly smaller day 7 and day 14 normalized tumor volumes.

Results

We examined the in vivo anti-cancer activity of the NP formulation of GMP-grade WHI-P131 in the MMTV/Neu transgenic mouse model of HER2 metastatic breast cancer. At a 50 mg/kg dose level, WHI-P131[NP] (like WHI-P131-free vehicle or PBS) did not exhibit significant in vivo anti-tumor activity capable of preventing tumor progression. However, at 100-150 mg/kg dose levels, WHI-P131 [NP] caused tumor shrinkage (Figure 1) and prevented the tumor growth. We applied an ANOVA model to compare the overall effect of control and drug treatments showing that 86% of the variation in tumor volumes was explained by the model at day 7 (p<0.0001) with a significant effect of treatment (F 4,183 = 7.813, P<0.0001) taking into account the effect of differences in tumor volumes at day 1 (F 2,209 = 388, P<0.0001). Examination of the ANOVA model at 14 days showed that 64% of the variation was explained (P<0.0001) with significant effects of treatment (F 4,164 = 9.755, P<0.0001), day 1 volume (F 1,208 = 141, P<0.0001) and day1*treatment interaction (F 4,205 = 3.509, P=0.009). Since there were significant effects for day 1 tumor volumes for both 7 and 14 day treatments and significant treatment effects accounting for these observed differences in day 1 measurements, we normalized all tumor volumes to day 1 measurements for statistical comparisons using T-tests of specific treatment groups. Specific comparisons of WHI-P131 [NP] with other drug treatments showed that it was significantly more effective than paclitaxel, gemcitabine, or gefitinib at the applied dose levels and treatment schedules (p<0.0001 for all comparisons), as documented by the ANOVA models performed at days 7 and 14 normalized tumor volumes.
in the WHI-P131 [NP] treatment group compared to other groups (Figure 2, Table 1). As shown in Figure 2 and Table 1, there was a significant decrease in tumor volume and arrest of tumor growth for WHI-P131[NP] treated mice (normalized volumes: 0.77±0.04 on day 7, P=7.5x10\(^{-9}\) and 0.70±0.06, on day 14, P=1.5x10\(^{-7}\) and continuation of growth for the other three drug treatments. While the tumor sizes consistently increased between days 7 and 14 for control mice, tumor shrinkage was observed in some of the WHI-P131 [NP] treated mice (Figure 2). It is noteworthy that the initial tumor volumes in the WHI-P131 [NP] treated test group were significantly larger than in the control group or chemotherapy group (1004±98 mm\(^3\) vs. 675±60 mm\(^3\) (Control) and 518±32 mm\(^3\) (Chemotherapy) (Table 1). Taken together, these results illustrate that GMP-grade WHI-P131 has promising in vivo anti-cancer activity in this chemotherapy-resistant breast cancer model when used as a nanoparticle formulation.

**Discussion**

Liposomal nanoparticle therapeutics containing cytotoxic agents may provide the foundation for potentially more effective and less toxic anti-cancer treatment strategies due to their improved pharmacokinetics, reduced systemic toxicity, and increased intratumoral/intracellular delivery [8,9]. Here we report the anti-cancer activity of a PEGylated nanoparticle formulation of GMP-grade WHI-P131 in the MMTV-neu transgenic mouse model of chemotherapy-resistant breast cancer. Notably, this therapeutic nanoparticle formulation of GMP-grade WHI-P131 was substantially more effective than the standard chemotherapy drugs paclitaxel, gemcitabine, and gefitinib against chemotherapy-resistant breast cancer in the MMTV/Neu transgenic mouse model. These findings demonstrate that the nanotechnology-enabled delivery of GMP-grade WHI-P131 shows potential for treatment of breast cancer.

Overexpression of ErbB2 (Her-2/neu) is associated with chemotherapy resistance and poor treatment outcome in breast cancer [10,29]. Chemotherapy resistance of ErbB2/Her2\(^\ast\) breast cancer cells has been attributed to activation of phosphatidylinositol 3 kinase (PI3-Kinase)/AKT anti-apoptotic signaling pathway and amplified expression of the resistance-associated survivin protein [10,29]. Use of the humanized recombinant monoclonal antibody trastuzumab/Herceptin binding the extracellular domain of the ErbB2/HER-2 receptor results in decreased chemoresistance and improved treatment outcome of ErbB2/HER-2\(^\ast\) breast cancer [17]. Our findings provide unprecedented evidence that the multifunctional tyrosine kinase inhibitor WHI-P131 is an active agent against chemotherapy-resistant ErbB2/HER-2\(^\ast\) breast cancer in the well-established MMTV-neu transgenic mouse model.

**References**


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<tr>
<th>Treatment Group</th>
<th># of Mice</th>
<th># of Tumors (Mean ± SEM)</th>
<th>Tumor Volume, mm(^3)</th>
<th>Normalized Tumor Volume (Mean ± SEM)</th>
<th>T-test P-Value vs Control</th>
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<tr>
<td>A. Control</td>
<td>38</td>
<td>65</td>
<td>60 ± 750 ± 64 ± 1095 ± 102</td>
<td>1.22 ± 0.06 ± 1.86 ± 0.19</td>
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<td>B. WHI-P131[NP]</td>
<td>17</td>
<td>38</td>
<td>98 ± 755 ± 89 ± 518 ± 46</td>
<td>0.006 ± 0.969 ± 0.002</td>
<td>0.006 ± 0.04 ± 0.70 ± 0.06</td>
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<tr>
<td>C. Chemo</td>
<td>81</td>
<td>116</td>
<td>32 ± 518 ± 39 ± 748 ± 46</td>
<td>0.023 ± 0.125 ± 0.003</td>
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<td>C.1 Taxol</td>
<td>27</td>
<td>41</td>
<td>51 ± 526 ± 57 ± 788 ± 71</td>
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<td>34</td>
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<tr>
<td>C.3 Gefitinib</td>
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<td>28</td>
<td>47 ± 433 ± 62 ± 599 ± 83</td>
<td>0.002 ± 0.004 ± 3.0x10(^{-7})</td>
<td>0.11 ± 0.08 ± 1.35 ± 0.11</td>
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**Table 1: Anti-Cancer Activity of WHI-P131 Nanoparticles in the MMTV/Neu Transgenic Mouse Model of Metastatic HER2\(^\ast\) Breast Cancer.** WHI-P131 [NP] (100 mg/kg or 150 mg/kg) was administered i.p. daily for 5 consecutive days each week, x 2 weeks; Taxol (6.7 mg/kg) was administered i.p. on days 1, 3, and 5; Gemcitabine (33.7 mg/kg) was administered i.p on days 1 and 8; Gefitinib (75 mg/kg) was administered daily by gavage. Control group included mice that were treated with i.p. injections of WHI-P131-free vehicle, WHI-P131 [NP] at the suboptimal 50 mg/kg dose level, or PBS.


