Supplemental Information

A Phase II Clinical Trial of Ixabepilone (Ixempra®, BMS-247550, NSC 710428), an Epothilone B Analog, in Patients with Metastatic Renal Cell Carcinoma

Hui Huang MD[1,6]
Michael Menefee MD[2,6]
Maureen Edgerly RN[1]
Sen Zhuang MD PhD[3]
Herb Kotz MD[1]
Marianne Poruchynsky PhD[1]
Lyn Mickley[1]
Susan Bates MD[1]
Tito Fojo MD PhD[1,4,5]

[1]Medical Oncology Branch, National Cancer Institute, NIH, Bethesda, MD
[2]Division of Hematology and Oncology, Mayo Clinic, 4500 San Pablo Road Jacksonville, FL 32224
[3]Ortho Biotech Oncology Research & Development, Johnson & Johnson, 920 US Route 202, Raritan, NJ 08869
[4]Corresponding Author and [5]Request for reprints:
Medical Oncology Branch, NCI, NIH
Building 10, RM 12N226
900 Rockville Pike
Bethesda, MD 20892
Telephone: 301-402-1357
FAX: 301-402-0172
E-mail: tfojo@helix.nih.gov
[6]The order of the first two authors is arbitrary

The following supplemental information is provided:

1. Extent of response is independent of the tumor burden or the disease measured in the RECIST evaluation
2. Target engagement following administration of ixabepilone.
3. Results of VHL sequence analysis.

EXTENT OF RESPONSE IS INDEPENDENT OF THE TUMOR BURDEN OR THE DISEASE MEASURED IN THE RECIST EVALUATION

Supplemental Figure 1: Patients had extensive tumor burden at baseline with a median of 21.7 cm of tumor (sum of longest diameters of all tumors), of which 10.5 cm tumor were evaluated by RECIST. The waterfall plot of best tumor response in percentage change from baseline for all patients is shown in Supplemental figure 2. The graphs directly above the response graph depict the total tumor burden in each patient and the sum of the largest diameters of the lesions followed in assessing response using the RECIST criteria. Together with Figure 1, these graphs demonstrate that the extent of response is independent of the tumor burden or the disease measured in the RECIST evaluation.

TARGET ENGAGEMENT FOLLOWING ADMINISTRATION OF IXABEPILONE

Note: *Supplemental figure 2 has been previously published[1]. It is included here for the convenience of the reader. There is no intention to depict this as a new figure.*

Methods:

Fresh tumor biopsy samples were obtained from patients before and after treatment with ixabepilone in cycle 1. These fresh patient core biopsies were Dounce homogenized at 22°C in hypotonic lysis buffer [1 mmol/L $MgCl_2$, 2 mmol/L EGTA, 0.5% Nonidet P-40, 20 mmol/L Tris-HCl (pH 6.8), and containing protease inhibitors] and vortexed vigorously. The lysates were passed through 18 and 21 gauge needles before centrifugation for 1 min at 1,000 rpm, removing unlysed debris. Total protein concentrations in the lysates were determined using the Bio-Rad assay, and equal amounts of protein were loaded on SDS-PAGE gels for each pair of pre-treatment and post-treatment samples. The western blot was sequentially probed with antibodies to total and modified α-tubulins and with an antibody to actin. Equally loaded pre-treatment and post-treatment lysate pairs were separated by SDS-PAGE on 4% to 15% gradient gels.

Results and Discussion:

α-Tubulin, a basic unit of microtubules, can undergo several post-translational modifications after assembly into stabilized microtubules, including acetylation of lysine 40 and detyrosination of the c-terminal tyrosine, exposing the penultimate glutamic acid and the new c-terminal residue. These modifications have been observed in cell culture after the addition of agents that stabilize microtubule. We developed a straightforward and dependable assay to show tubulin target engagement in tumor tissue after

treatment of patients with ixabepilone (BMS-247550; Ixempra). Among the 13 patients that underwent two biopsies, we found increased levels of both acetylated- and glu-terminated-alpha tubulin after ixapebilone treatment in 84 to 92% of serial biopsies. We also found higher levels of total tubulin. After normalizing to total tubulin levels, the net increase of glu-terminated-alpha or acetylated alpha tubulins was still observed. The increase in total alpha tubulin occurs in response to reduced soluble $\alpha/ß$ dimers and is an adaptive response. These results provided evidence of target engagement by ixabepilone in our study. However, the results did not correlate with a clinical response. We conclude that microtubule target engagement is not sufficient enough to bring about cell death, but rather that additional events must occur downstream of this event and that these events either do not occur in the cancer cells or do not occur to an extent sufficient to bring about cell death. Alternately, the clinical response may depend on the extent of microtubule stabilization and the timing of the drug effects such that they coincide with the time at which the cells reach a vulnerable phase in the cell cycle.

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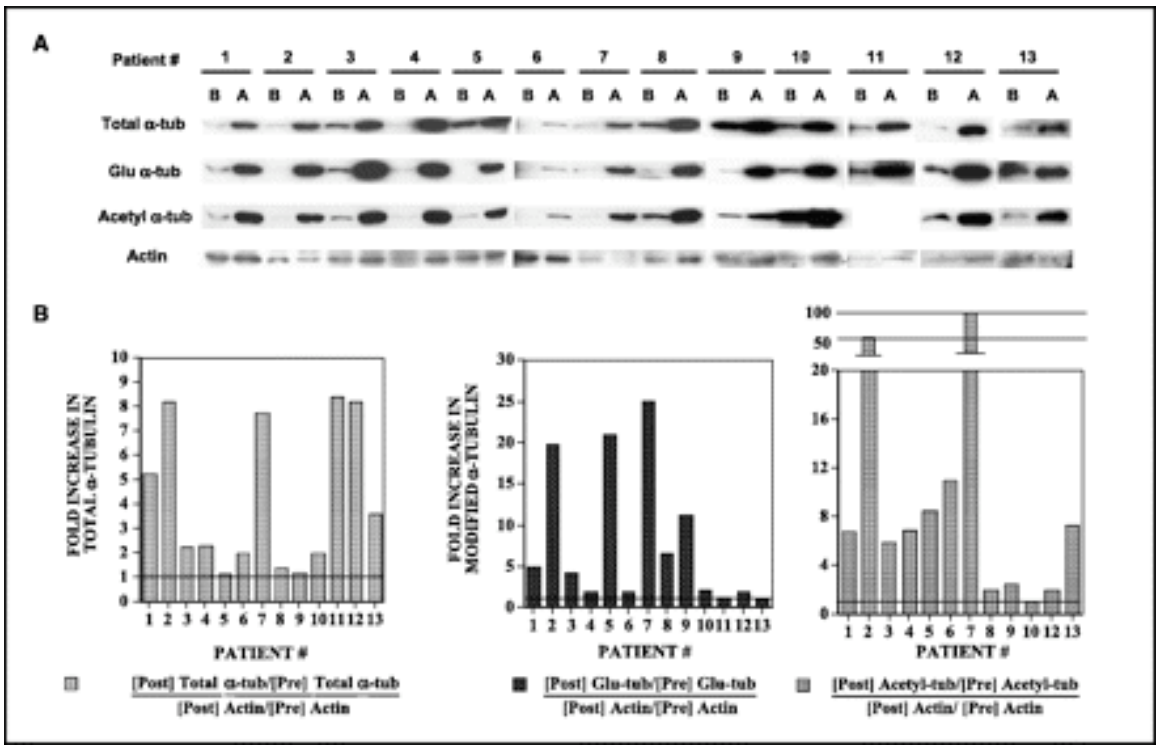


Supplemental Figure 2: Total α-tubulin and modified α-tubulin levels increased in serial tumor biopsies obtained from patients receiving ixabepilone (BMS-247550) treatment. A, tumor samples were obtained before treatment and after the fifth dose of ixabepilone (BMS-247550) given for five consecutive days at a dose of 6 mg/m2/d over 1 h. The samples were homogenized and lysed. Total protein concentrations in the lysates were determined, and equal amounts of protein were loaded on SDS-PAGE for each pair of before (B) and after (A) treatment samples. The Western blot was sequentially probed with antibodies to total and modified α-tubulins and with an antibody to actin, the latter serving as a control for loading. B, quantitation of A. Patient numbers are indicated on the X axis. Total α-tubulin and modified α-tubulin levels were normalized to actin levels[1].

RESULTS OF VHL SEQUENCE ANALYSIS

Background:

Based largely on theoretical considerations that VHL mutation status could affect the response to ixabepilone, a decision was made after some activity was observed early in the study to double the accrual, expecting that about one half of the patients would present with a mutation in the VHL gene – an estimate based on available data. The approach and results, described here included validation of the experimental procedure by sequencing 10 controls, whose mutations were not known to the individual performing the analysis, but were identified in every case.

Methods - RNA/DNA isolation:

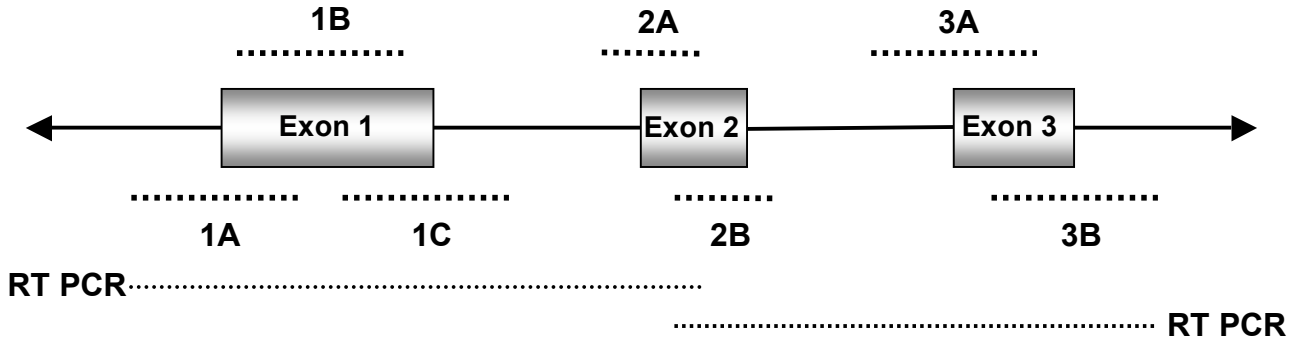
Sequencing of VHL was performed using either micro-dissected paraffin embedded or frozen tissue. Patient RNA was isolated from needle biopsy specimens using the Trizol (Invitrogen, Carlsbad, CA) extraction method. Cell line RNA was also isolated using Trizol. Patient DNA was isolated from paraffin embedded tissue after micro dissection. Tissue slices were extracted in 50 - 100 µl lysis buffer containing 0.5% Tween 20, 1 mM EDTA pH 8.0, 50 mM Tris-HCL pH 8.5 and 500 mg/ml proteinase K. Lysis and proteinase K digestion was carried out for 3 days at 56^{o}C. Fresh proteinase K was added on day 2 and day 3 at a final concentration of 500 mg/ml. After a 4-hour incubation with proteinase K on day 3, samples were heat inactivated at 95^{o}C for 10 min.

RT and PCR:

RNA samples were reverse transcribed in a 20 µl reaction using 1.0 µg RNA, 1 µl random primers (fc 50 ng), 0.7 µl MMLV reverse transcriptase (fc 140 units), 2 µl dNTP (fc 1mM) and 0.5 µl RNAse inhibitor (fc 20 units). Either 5 µl cDNA template or 2 µl DNA template (isolated from microdissected tissue slices as described above) were used in the PCR reaction. PCR products were generated using the primers listed in Table 1 and Platinum Taq High Fidelity DNA polymerase (Invitrogen, Carlsbad, CA) according to protocol instructions.

DNA Sequencing:

Cycle sequencing was performed on 2 µl of each PCR reaction using the sequencing primers listed in Table 1 and Big Dye 1.1 from ABI (Foster City, CA). Dye terminator removal was performed on all reactions using Performa DTR Gel Filtration cartridges (Edge Biosystems, Gaithersburg, MD). Samples were run on an ABI model 3100 DNA sequencer.



Supplemental Figure 3.
Short dotted lines: PCR products obtained from microdissected tissue.
Long dotted lines: RT PCR products from cell lines and needle biopsy samples.

SUPPLEMENTAL TABLE 1

Genomic Primers	Primer sequence	PCR product
Exon 1a 5'*	gggtggtctggatcgcggag	134 bp
Exon 1a 3'	actcctcggcgcccgactct	
Exon 1b 5'	cgtcgaagagtacggccctg	182 bp
Exon 1b 3'	ctgcgattgcagaagatgacc	
Exon 1c 5'	gaactcgcgcgagccctccc	179 bp
Exon 1c 3'	tcccctgctgggtcgggccta	
Exon 2a 5'*	cgtgcccagccaccggtgtg	140 bp
Exon 2a 3'*	gtcaacattgagagatggca	
Exon 2b 5'	ggacacacgatgggcttctggt	153 bp
Exon 2b 3	ggtctatcctgtacttaccacaac	
Exon 3a 5'	cgttccttgtactgagaccctag	170 bp
Exon 3a 3	tgggtggtcttccagatcttcg	
Exon 3b 5'	caggagactggacatcgtcagg	162 bp
Exon 3b 3'*	ccatacaaaagctgagatgaaacagtg	
*RNA Primers		

Sequencing	Primer sequence	
1a5'seq	gtctggatcgcggagggaatg	
1a3'seq	cccgactcctccccgcct	
1b5'seq	tacggccctgaagaagacgg	
1b3'seq	ggagggctcgcggagttca	
1c5'seq	caggtcatcttctgcaatcg	
1c3'seq	ccctaagcgccgggccc	
2 5'seq	ccaccggtgtggctctttaac	
2 3'seq	acttaccacaacaaccttat	
3 5'seq	gtactgagaccctagtctgtc	
3 3' seq	tgagatgaaacagtgtaagt	

Results and Discussion:

The coding sequence of the VHL gene in 32 patients with metastatic renal cell carcinoma (mRCC) was sequenced for mutations. The primers used in the PCR and sequencing reactions are listed in Supplemental Table 1 and shown schematically in Supplemental Figure 3. A needle biopsy of patient tumor was obtained from 14 patients. RNA was isolated from these patients and RT PCR was performed as described above. Microdissection of paraffin embedded tumor obtained from 20 patients was performed so as to isolate DNA and this was followed by PCR as described above. DNA isolated from the A498 cell line was used as a positive control for PCR and sequencing of the micro dissected tissue samples. We obtained both RNA and DNA from 2 patients. We detected a VHL mutation in only 1 of 32 patient samples. A 1 bp deletion at nucleotide 317 (relative to start of translation) resulted in a premature stop codon at amino acid 158.

Because previous reports have cited a high frequency of mutations in the VHL gene in RCC patients [2-5] we wanted to ensure we could detect known mutations in the VHL gene using our methods. We thus isolated DNA from 8 RCC cell lines obtained from both the NCI Drug Screen and the Urologic Oncology Branch of the NCI. In addition, DNA was isolated from 3 RCC tumors also obtained from the Urologic Oncology Branch of the NCI and 3 pheochromocytoma tumors from patients diagnosed with VHL obtained from the Reproductive Biology and Medicine Branch of the NICHD. Sequencing of RT PCR products from these cell lines and tumors identified the mutations listed in Supplemental Table 2. With the exception of the pheochomocytoma

tumors all mutations had been previously described or confirmed by the laboratories that provided the samples. It was felt that this ability to detect known mutations with our approach and our primers validated the methodology used and established its sensitivity. We would note that sequencing of untranslated exon 1 was not performed and that the methylation status of the samples was not analyzed. Perhaps the VHL mutation rate in our samples would have been higher had we analyzed the samples for these changes, but it was clear that the data indicated a low frequency of VHL mutations in this cohort of patients with sporadic RCC.

SUPPLEMENTAL TABLE 2

Source of	VHL Mutation
Cell Lines	
UO31*	WT
Caki*	WT
TK10*	WT
SN12C*	WT
A498*	Exon 2 – 4 bp deletion (GTCA): early stop @
UOK 150**	Exon 1 – aa 98 Tyr (**t**ac) to Asn (**a**ac)
UOK 161**	Exon 3 – aa 164 Gln (**c**ag) to Stop (**t**ag)
786-0*	Exon 1 – Deletion at NT 311: early stop at aa 158
RCC Tumors	
126T	Exon 3 – aa 162 Cys (**t**gc) to Arg (**c**gc)
142T	6 bp del from NT361-366 deleting aa 121 and 122
161T	Exon 3 – aa 164 Gln (**c**ag) to Stop (**t**ag)
Pheochromocytomas	
S34	Exon 3 – aa 185 Tyr (t**a**c) to Phe (t**t**c)
33V	Exon 2 – aa 136 Phe (t**t**t) to Cys (t**g**t)
V34A	Exon 3 – aa 167Arg (c**g**g) to Gln (c**a**g)

*Cell lines obtained from the NCI Drug Screen

Supplemental References:

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