ARRY-520, a Novel KSP Inhibitor with Potent Activity in Hematological and Taxane-resistant Tumor Models

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Abstract. *Aim: Profiling the efficacy and pharmacodynamic activity of the kinesin spindle protein (KSP) inhibitor ARRY-520 will aid the identification of responsive tumor types and pharmacodynamic profiles that correlate with activity. Materials and Methods: In vivo activity was evaluated in a diverse panel of 16 different tumor xenograft models. Pharmacodynamic activity was evaluated in selected models. Results: ARRY-520 had low nanomolar antiproliferative activity in tumor cell lines. Monopolar spindles were formed at active potencies. Partial or complete responses were observed in 13/16 xenograft models. Hematological tumors were particularly sensitive, with a 100% complete response rate in some models. Maintenance of mitotic block for a sufficient length of time for cells to lose survival signals and progress to apoptosis was a key component of the mechanism of activity. ARRY-520 was also active in several taxane resistant models. Conclusion: The data provide a rationale for clinical evaluation of the activity of ARRY-520 in hematological carcinomas and taxane-resistant tumors.*

Anti-mitotic therapy has proven clinical benefit in the treatment of cancer. Taxanes and epothilones, which stabilize microtubule polymers, and vinca alkaloids, which inhibit tubulin polymerization, are used to treat a variety of carcinomas, including breast, ovarian, lung and leukemias (1). A disadvantage of the anti-mitotic therapies in current clinical use is the mechanism based toxicity arising from the disruption of microtubule dynamics in cellular processes not involved in proliferation. Taxanes, vinca alkaloids and epothilones do not distinguish between microtubule-dependent subcellular transport in synaptic vesicles or Golgi apparatus and mitosis. This lack of specificity leads to neurotoxicity, which can be dose-limiting with these agents (2, 3).

Kinesin spindle protein (KSP, eg5), a member of the mitotic kinesin family, is involved in the early stages of mitosis. KSP is responsible for centrosome separation, which is required for the formation and maintenance of the bipolar spindle. Inhibition of KSP activity blocks cells in mitosis resulting in the formation of a monopolar spindle. The inhibition of KSP activity is an attractive chemotherapeutic approach because use of such inhibitors may avoid negative effects on microtubule dynamics in non-target cells, such as post mitotic neurons, where KSP is not expresssed (2, 3). This appears to be the case, as no instances of peripheral neuropathy have been reported for the KSP inhibitors that have been administered in human clinical trials (3). Because KSP inhibition is a novel mechanistic approach to anti-mitotic therapy, with potentially unique mechanisms of resistance, it is also possible that KSP inhibitors will show activity in tumors that are refractory to standard therapies.

KSP inhibitors have so far shown only modest activity in early clinical trials (4-7). To improve the response, we have taken the two-pronged approach of designing a potent and broadly active inhibitor and using translational research to identify appropriate tumor settings to optimize the chance for clinical success. ARRY-520 is a potent inhibitor of KSP, with an IC_{50} of 6 nM, and is highly selective, with an IC_{50} for inhibition of eight other kinesins greater than 100 μM and no significant inhibition of a panel of 224 kinases at 10 μM (8). The molecule is noncompetitive with ATP and with microtubules (8) and binds to KSP at the same site as monastrol, an allosteric inhibitor of the enzyme (9), confirmed by X-ray crystallography (8). Here the *in vivo* efficacy and pharmacodynamics of ARRY-520 are described.

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Table I. *Antitumor activity in subcutaneous xenograft models. Mice were treated with ARRY-520, q4dx3.*

Xenograft (tumor type)	Dose (mg/kg)	Day of measurement	% PD	% SD	% PR	% CR	% Cure (day)	% TGI
HT-29 (colon)	30	23		12	75	12	25 (47)	
HCT-116 (colon)	30	26		20	80			
HCT-15 (colon)	30	15	100					45
A2780 (ovarian)	27	23			60	40		
LOX-IMVI (melanoma)	30	10	100					95
MDA-MB-231 (breast)	20	8		12	63	25		
MDA-MB-468 (breast)	30	37	100					78
UISO-BCA-1 (breast)	20	15		37	63			
DU145 (prostate)	30	12		50	50			
PC-3 (prostate)	30	12			100			
RPMI8226 (multiple myeloma)	20	26				100	33 (71)	
JJN3 (multiple myeloma)	20	15		50	38	12		
H929 (multiple myeloma)	20	32	12	12	25	50		
MV4-11 (AML)	20	28				100	25 (50)	
HL-60 (APML)	27	15				100		
K562 (CML)	27	16		20	60	20		

PD: Progressive disease, tumor volume greater than 150% of volume at start of treatment, SD: stable disease, tumor volume 50-150% of volume at start of treatment, PR: partial response, tumor volume less than 50% of the volume at start of treatment, CR: complete response, no detectable tumor, Cure: long term complete response. %TGI: % tumor growth inhibition calculated on the day when average volume of the vehicle control group first exceeded 2,000 mm^3: 0% TGI is the volume of the vehicle control group at the day of measurement, and 100% TGI is the volume at the start of treatment; AML: acute myelogenous leukemia, APML: acute promyelocytic leukemia, CML: chronic myelomgenous leukemia.

Materials and Methods

Cell lines and culture. HeLa (cervical), HT-29, HCT-15 and HCT-116 (colon), A2780 and NCI/ADR-RES (ovarian), DU145 and PC-3 (prostate), SKBR3, MDA-MB-231, MDA-MB-468 and MCF-7 (breast), A431 (epidermoid), B16 (melanoma), C6 (glioma), RPMI8226 and H929 (multiple myeloma), MV4-11 (AML), HL-60, KU182, KG1, Molt3, Molt4, K562 and K562/ADR (leukemia) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). LOX-IMVI (melanoma) cells were obtained from the National Cancer Institute cell bank (Frederick, MD, USA). JJN3 (multiple myeloma) cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The cells were grown under the conditions recommended by the suppliers.

In vitro assays. Measurements of the inhibition of enzyme activity, cell proliferation, mitotic arrest and cellular imaging were performed as described previously (8).

Compounds and formulations. ARRY-520 was synthesized at Array BioPharma, and formulated in either 25% polyethylene glycol (PEG)-400/10% EtOH / 65% normal saline or 100% normal saline. The plasma pharmacokinetics of ARRY-520 in female nude mice were the same in both vehicles (data not shown). Paclitaxel was purchased from Hauser Pharmaceuticals (Denver, CO, USA) or Astatech (Bristol, PA, USA) and formulated in 10% EtOH / 10% Cremophor EL (cat. # 51635553; BASF, Ludwigshafen, Germany) / 80% saline. Docetaxel was purchased from Sanofi-Aventis (Bridgewater, NJ, USA) as a 40 mg/ml solution in polysorbate 80, and diluted to the appropriate concentration with 13% EtOH in saline.

In vivo xenograft models. All the studies were performed in accordance with the Array BioPharma Institutional Animal Care and Use Committee guidelines and in harmony with the Guide for Laboratory Animal Care and Use. Immunocompromised mice were purchased from either Charles River (Wilmington, MA, USA) or Taconic (Germantown, NY, USA). Tumor cells ($2\text{-}20\times10^6$) were injected into the immunocompromised mice subcutaneously (*s.c.*) in the right flank. Solid tumor xenografts (HT-29, HCT-116, HCT-15, A2780, LOX-IMVI) were grown in female nude mice. Hematological tumor xenografts were grown in either female severe combined immunodeficient (SCID)-beige mice (RPMI8226, JJN3, H929, K562, HL-60) or female non-obese diabetic (NOD)-SCID mice (MV4-11). PC-3 and DU145 xenografts were grown from cells implanted *s.c.* in male nude mice. MDA-MB-231, MDA-MB-468 and UISO-BCA-1 xenografts were grown from serially transplanted tumor fragments implanted *s.c.* in female nude mice using a trocar needle. The UISO-BCA-1 tumor line was derived from a pleural effusion, and is estrogen receptor negative, progesterone receptor negative, and HER2 positive (10). The tumors were allowed to grow to a volume of ~150-250 mm^3 (PC-3 and DU145 tumors were grown to ~90 mm^3), and the mice were randomized, based on tumor size, into groups of 5-8. All the compounds were administered *i.p.* in a volume of 10 ml/kg, unless otherwise indicated. Tumor volumes and animal weights were measured two or three times weekly after the initiation of treatment. Tumor volumes were determined by measuring the width and length of the tumor with calipers and applying the formula, volume = $(\text{width}^2 \times \text{length})/2$.

In vivo pharmacodynamic experiments. Subcutaneous tumor xenografts were allowed to grow to a volume of 250-350 mm^3. The mice were randomized into groups of 3-4 based on tumor size, and were given a single dose of ARRY-520 *i.p.* At various time-points

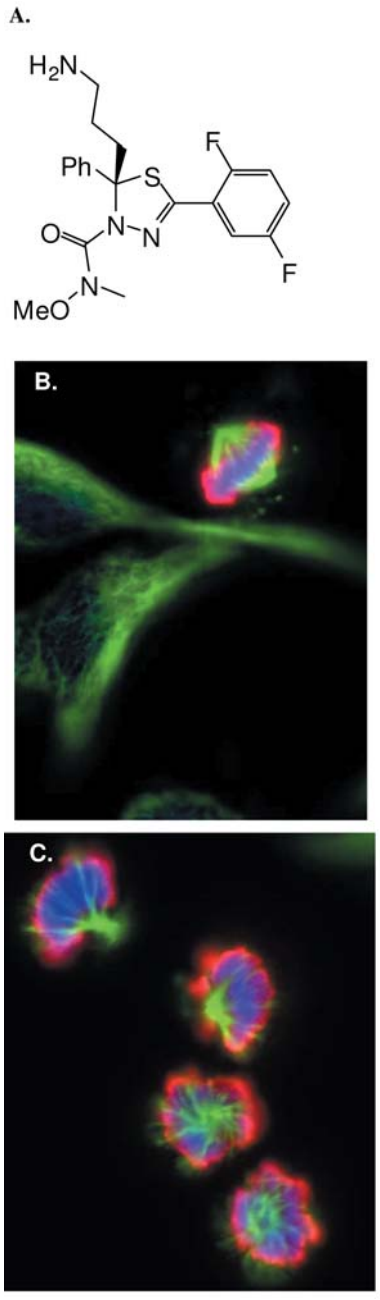

Figure 1. *A: Chemical structure of ARRY-520. B and C: Fluorescence microscopy of HeLa cells after 16 hours of incubation with 0.5% DMSO (B) or 10 nM ARRY-520 (C), followed by fixation and incubation with antibodies to α-tubulin (green), pHH3 (red) and DNA intercalator Hoechst 33342 (blue).*

after administration of the drug, the mice were euthanized by CO_2 inhalation and the tumors excised and placed in 10% neutral buffered formalin. The formalin-fixed tumors were processed and paraffin embedded by standard procedures. Spindle morphology was analyzed by staining tumor sections for α-tubulin, and apoptosis was analyzed by TUNEL stain, as described previously (11). Monopolar/abnormal spindles and TUNEL positive (apoptotic) cells were counted in three x40 fields from each sample, analyzed using algorithms developed in ImagePro software (Media Cybernetics, Inc., Bethesda, MD, USA), and data reported as mean±SEM.

Results

Cell proliferation and mitotic arrest. The EC_{50}s for inhibition of cell proliferation ranged from 0.4 to 3.1 nM in HeLa, HT-29, HCT-116, A2780, LOX-IMVI, B16, C6, MDA-MB-468, MDA-MB-231, SKBR3, MCF-7, DU145, PC3, A431, RPMI8226, JJN3, H929, MV4-11, HL-60, K562, KU812, KG1, Molt3 and Molt 4 cell lines. EC_{50}s for mitotic arrest, measured as the concentration of ARRY-520 required for 50% of the maximum elevation in phosphorylation of serine 10 of histone H3 (pHH3), were in the same range.

ARRY-520 retained activity in multidrug-resistant cell lines. HCT-15, NCI/ADR-RES (ovarian) and K562/ADR (leukemia) cell lines have elevated P-glycoprotein (PgP) activity (12-15), and are resistant to clinically used cancer therapeutics known to be PgP substrates, such as paclitaxel. The EC_{50} of ARRY-520 for inhibition of proliferation of HCT-15, NCI/ADR-RES and K562/ADR cells was 3.7, 14 and 4.2 nM respectively, while the EC_{50}s of paclitaxel in these cell lines was 35, 565 and 372 nM. K562/ADR was 118-fold resistant to paclitaxel compared to the parent K562 line, but only 3.5-fold resistant to ARRY-520.

Immunohistochemical (IHC) analysis of cells arrested in mitosis by ARRY-520. The ability of ARRY-520 to disrupt mitosis and display the monopolar phenotype characteristic of KSP inhibition was demonstrated in HeLa cells using cellular imaging. The majority of cells treated with vehicle for 16 hours were in interphase, with a few cells captured in normal mitosis, with a typical bipolar spindle and DNA aligned on the metaphase plate (Figure 1B). After treatment with 10 nM ARRY-520 for 16 hours, a majority of cells were observed to be blocked in mitosis with the monopolar spindle structure typical of KSP inhibition (Figure 1C).

In vivo efficacy. The maximum tolerated dose (MTD) of ARRY-520 dosed *i.p.*, *q4d*x3 was 25-30 mg/kg in female nude mice and 20-27 mg/kg in female SCID-beige mice. MTD is defined as the dose where none of the mice had weight loss greater than 20% of initial body weight and no other unacceptable drug-related tolerability issues during the course of treatment and recovery.

The efficacy and dose response for ARRY-520 was evaluated in female nude mice bearing subcutaneous HT-29 xenografts. Treatment over a range from 10 to 30 mg/kg indicated that the minimally effective dose on the *q4d*x3 schedule was between 15 and 20 mg/kg, with a sharp dose response (Figure 2A). Response rates at the 30 mg/kg dose are shown in Table I. The antitumor activity of ARRY-520 was

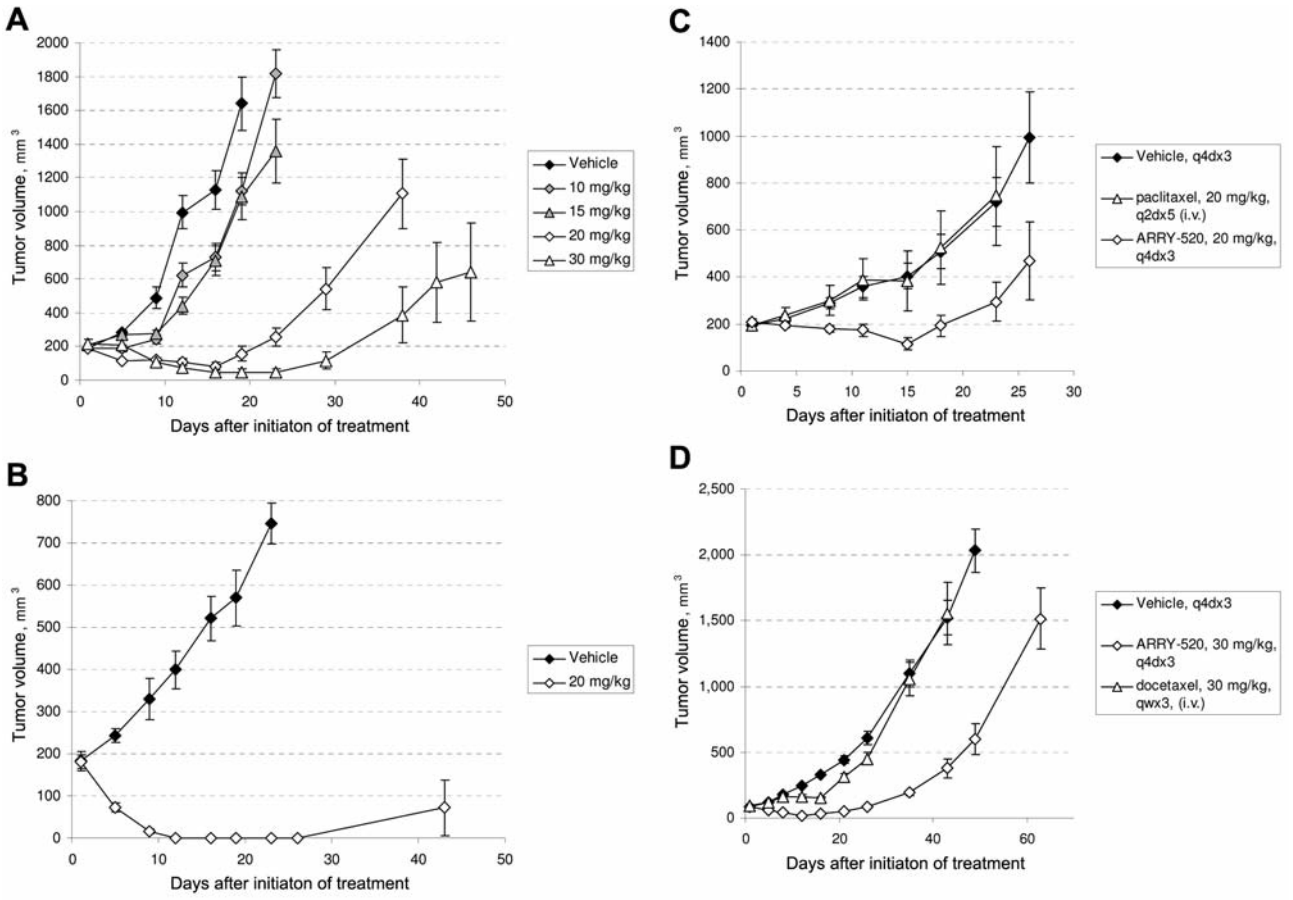

Figure 2. *Tumor volume of subcutaneous HT-29 (A) and RPMI8226 (B) xenografts after treatment with ARRY-520 or vehicle, i.p., q4dx3, and tumor volume of UISO-BCA-1 (C) and PC-3 (D) xenografts after treatment with ARRY-520, paclitaxel or docetaxel. Treatment began at day 1. Error bars represent SEM.*

subsequently evaluated in 15 additional xenograft models, including colon, ovarian, melanoma, breast and prostate models of solid tumors; and multiple myeloma, CML and AML models of hematological tumors (Table I). For tumors with complete responses, partial responses or stable disease, the percentage response rates are reported. For the xenograft models where progressive disease was the best response, the percentage tumor growth inhibition is reported. Treatment with doses at or near the MTD resulted in a high percentage of partial responses, complete responses and cures in most models, with the exception of HCT-15, LOX-IMVI and MDA-MB-468. The hematological carcinomas were particularly sensitive to ARRY-520, with a 100% complete response rate for MV4-11, HL-60 and RPMI8226 xenografts, including long-term complete responses with RPMI8226 and MV4-11. Growth curves for RPMI8226 xenografts from vehicle treated and ARRY-520 treated mice are shown in Figure 2B.

The antitumor efficacy of ARRY-520 was compared to that of paclitaxel or docetaxel in breast, colon, ovarian and prostate cancer xenograft models that are resistant or refractory to taxanes. ARRY-520 was active in UISO-BCA-1 xenografts (Figure 2C), which were completely resistant to paclitaxel. The antitumor efficacy of ARRY-520 was also superior to paclitaxel in mice bearing subcutaneous HT-29, HCT-116, MDA-MB-231 and A2780 xenografts. HCT-15, an extreme overexpressor of PgP activity (12) was unresponsive to paclitaxel at its maximum tolerated dose (MTD) (30 mg/kg) on a *q4dx*4 schedule, but showed modest sensitivity to ARRY-520 (data not shown). ARRY-520 was superior to docetaxel in the androgen receptor-negative prostate cancer xenograft model PC-3 (Figure 2D), and was also superior to docetaxel in the DU145 prostate xenograft model.

In vivo pharmacodynamics. As described above, the RPMI8226 xenografts were noticeably more sensitive to the antitumor activity of ARRY-520 than were the HT-29 xenografts (Figure 2A and B, Table I). In order to understand the biological basis for differences in tumor response to ARRY-520, the pharmacodynamic response was compared in these two tumor types.

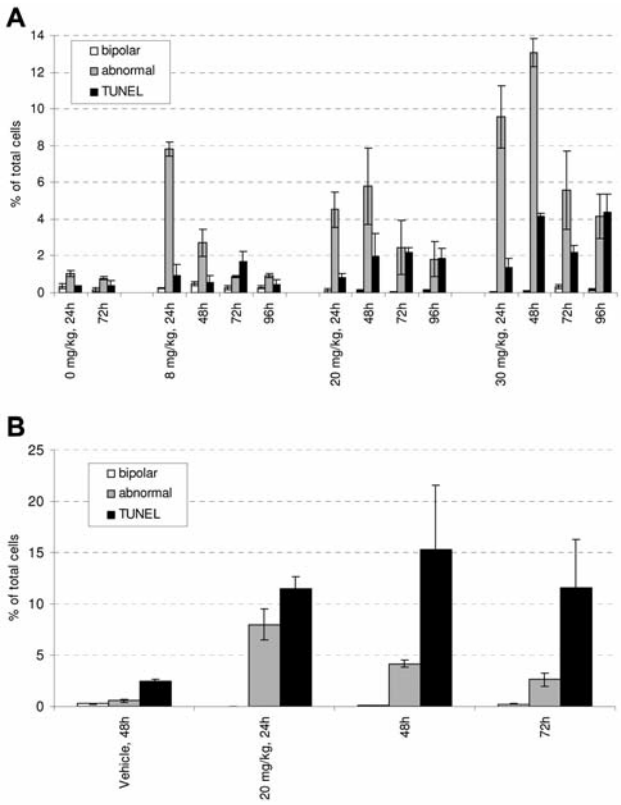

Figure 3. *Quantification of the percentage of cells containing bipolar spindles, abnormal spindles, and percentage of cells staining positive for TUNEL in IHC stained sections from subcutaneous HT-29 (A) or RPMI8226 (B) xenografts taken from mice after a single treatment with ARRY-520. Error bars represent SEM.*

The percentage of cells with abnormal spindles (*i.e.*, monopolar + abnormal) in the HT-29 xenografts was measured up to 96 hours (4 days, the dosing interval on the *q*4*d*x3 schedule) after drug treatment at doses of 8, 20 or 30 mg/kg (Figure 3A). At the non-efficacious dose of 8 mg/kg, the percentage of cells with abnormal spindles returned to baseline (vehicle control) levels by 72 hours after dosing. At efficacious doses (20 and 30 mg/kg), an elevated percentage of cells with abnormal spindles persisted through 96 hours. The percentage of cells with abnormal spindles was greatest at the 30 mg/kg dose, with a 13-fold increase over baseline (vehicle control) at 48 hours, and a 5-fold increase persisting for 96 hours. Consistent with these observations, the percentage of cells staining positive for TUNEL dropped to baseline by 96 hours after dosing at 8 mg/kg, but was maintained at levels 5-fold and 10-fold above baseline after dosing at 20 and 30 mg/kg, respectively (Figure 3A). Thus, the antitumor activity of ARRY-520 in the HT-29 xenografts was associated with sustained arrest of mitosis and the subsequent onset of apoptosis.

Interestingly, the maximum percentage of cells with abnormal spindles in the RPMI8226 xenografts from mice treated with ARRY-520 at MTD (Figure 3B), was slightly lower than that seen in the HT-29 xenografts at MTD (8% *vs*. 12%). However, the maximum percentage of cells staining positive for TUNEL in the RPMI8226 xenografts was noticeably higher (15% *vs*. 4%). Also of note, a greater percentage of TUNEL-positive cells was observed in the RPMI8226 xenografts by 24 hours (10% *vs*. 1%), suggesting an earlier onset of apoptosis in RPMI8226, compared to HT-29. Thus, the greater antitumor activity of ARRY-520 in the RPMI8226 multiple myeloma xenografts was associated with a much higher level of apoptosis sooner after drug treatment, despite a slightly lower percentage of abnormal spindles, suggesting that these cells are intrinsically more sensitive to the induction of apoptosis after mitotic block mediated by a KSP inhibitor. Representative images of IHC staining for α-tubulin and TUNEL in RPMI8226 xenografts are shown in Figure 4.

Discussion

When ARRY-520 was administered on a *q*4*d*x3 schedule to mice bearing subcutaneous xenografts, the compound was active in all 16 models tested, with partial or complete responses observed in 13 of the models. The hematological carcinomas were particularly sensitive to ARRY-520, with a 100% complete response rate in the RPMI8226, MV4-11 and HL-60 xenografts, including long-term complete responses (greater than 7 weeks) in the RPMI8226 and MV4-11 xenografts. Regression of RPMI8226 xenografts was clearly evident as early as four days after the first dose of ARRY-520, and was accompanied by a maximum percentage of apoptotic cells of 15% and a peak in abnormal spindle count at 24 hours. In contrast, significant regression of the HT-29 xenografts in mice dosed with ARRY-520 occurred more slowly, the maximum percentage of apoptotic cells reached only 4% and an elevated abnormal spindle count had to be maintained for 72-96 hours to achieve antitumor activity. These observations suggested that tumors requiring a shorter mitotic block before entry into apoptosis, such as RPMI8226, will be more sensitive to treatment with ARRY-520. The time-course of apoptosis after treatment with ARRY-520 *in vitro*, as shown by the maximum elevation of caspase-3 activity occurring at 24 hours for RPMI8226 cells treated with ARRY-520 in cell culture, but not until 72 hours for HT-29 cells (16) was consistent with these *in vivo* observations:

When a cell is blocked in mitosis, there is no new protein synthesis occurring, so the cell fate depends on a combination of the length of the mitotic block, the half-life of the survival protein(s) and the half-life of the mitotic checkpoint proteins. The work of Shi *et al*. (17), Gascione *et al*. (18), Blagosklonny (19) and and Bekier *et al*. (20)

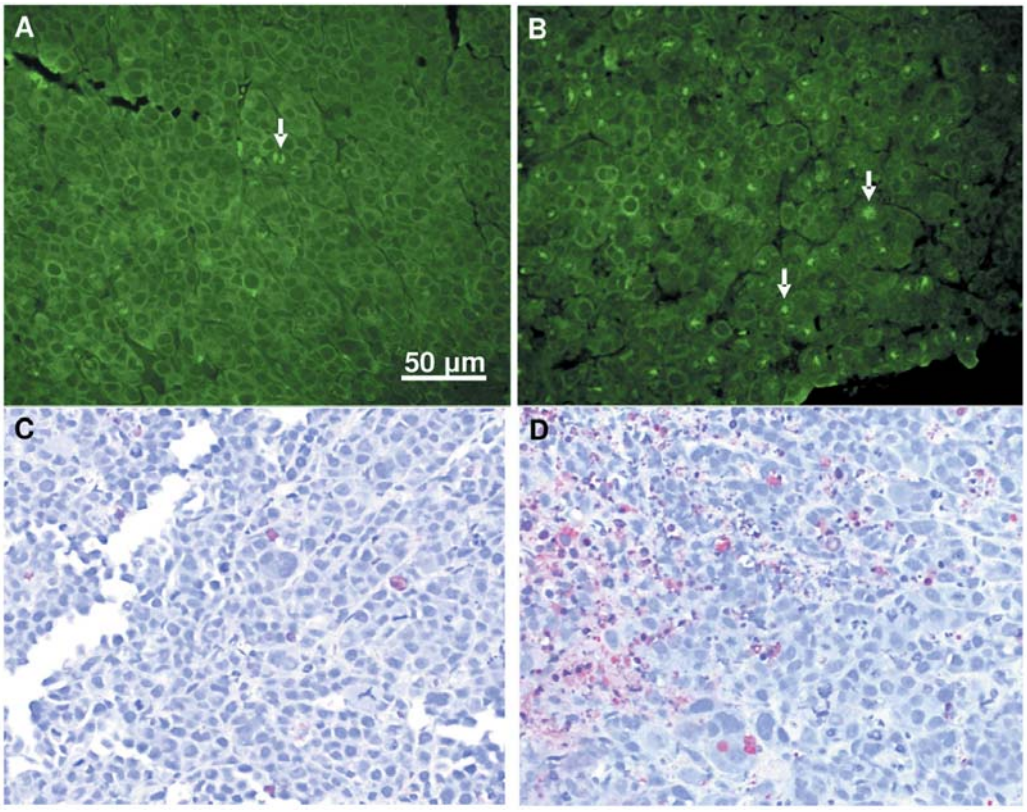

Figure 4. *Representative images of α-tubulin (A, B) and TUNEL (C, D) in IHC stained sections from subcutaneous RPMI8226 xenografts taken from nude mice 24 hours (A, B) and 48 hours (C, D) after a single treatment with vehicle (A, C) or 20 mg/kg ARRY-520 (B, D). The arrow in panel A indicates a cell with a bipolar spindle. The bright spots in B are the condensed chromatin associated with monopolar or abnormal spindles; two examples are indicated by the arrows. The scale bar in panel A applies to all images.*

suggests three possible outcomes after treatment with a mitotic inhibitor: i) the mitotic block is not maintained long enough for loss of survival proteins to trigger apoptosis or loss of checkpoint proteins to trigger progression into interphase and the cell progresses in the cell cycle normally; ii) the mitotic block is maintained long enough for the level of survival proteins to drop below the threshold required for biological activity, but the checkpoint is maintained and the cell progresses into apoptosis while still in mitosis, or iii) the mitotic block is maintained long enough for the level of checkpoint proteins to drop below the threshold required for biological activity, but the survival signal is maintained, and the cell progresses into interphase with a damaged chromosome and may die in interphase or at the next mitotic cycle. This model provides a rationale for correlating the antitumor activity of ARRY-520 with mitotic block and progression into apoptosis in the HT-29 and RPMI8226 xenografts.

Regulation of survival and apoptosis is a complex process. Survival proteins such as mcl-1, bcl-2 and bcl-XL prevent apoptosis (21), while spindle checkpoint proteins such as cyclin B, BubR1, Mad2 and survivin (22-26) prevent cells from exiting mitosis. Different tumor cell lines are dependent on different survival proteins as their primary survival signal. Tumor cells dependent on survival proteins with a short half-life, such as mcl-1 (27, 28), should require a shorter mitotic block before entering apoptosis (outcome ii above) while cells dependent on survival proteins with a longer half-life, such as bcl-2 or bcl-XL, should require a longer mitotic block before the onset of apoptosis, and may enter apoptosis as described in outcome iii. Multiple myeloma cells, including RPMI8226 (as well as hematological carcinomas in general), are frequently dependent on mcl-1 for survival (29-31). The hypothesis that mcl-1 plays a key role in the sensitivity of multiple myeloma cells to ARRY-520 is supported by recent work from our laboratory (16), in which the sensitivity of JJN3 and H929 multiple myeloma cells to apoptosis induced by ARRY-520 was altered by changing the level and stability of mcl-1 protein. Increasing the expression or stability of mcl-1 increased the time required for apoptosis induction and decreasing the expression or

stability of mcl-1 decreased the time required for apoptosis induction by ARRY-520. The *in vivo* observations reported here, indicating that multiple myeloma tumors are particularly sensitive to ARRY-520, were consistent with these results. Furthermore, Western blot analysis of HT-29 and RPMI8226 xenografts showed a 7-fold higher level of mcl-1 protein in RPMI8226 *vs*. HT-29 xenografts, and a 3-fold higher level of bcl-XL in HT-29 *vs*. RPMI8226 xenografts (R Woessner, unpublished results).

Also of note was the potent activity of ARRY-520 in tumor lines that were unresponsive or poorly responsive to taxanes. This highlighted the potential advantages of targeting tumors with a novel antimitotic agent. Firstly, inhibition of KSP has the potential to obtain the therapeutic efficacy of mitotic inhibitors such as the taxanes, but without the peripheral neuropathy associated with taxane treatment (1-3). The experience to date with KSP inhibitors in the clinic shows no evidence of peripheral neuropathy. Toxicological studies of ARRY-520 in rats and dogs also showed no evidence of neuropathic effects. Secondly, blocking mitosis by a non-tubulin targeted mechanism has the potential for efficacy in cases where the cancer is refractory or has become resistant to taxane therapy. Although the role of the multidrug resistant phenotype in taxane resistance in the clinic is unclear, the results reported here suggested that ARRY-520 may be active in settings of taxane resistance or failure where multidrug resistance plays a role.

Characterization of the pharmacodynamic activity of ARRY-520 suggested that maximal activity was associated with prolonged mitotic inhibition, and that tumors which require a shorter mitotic block before entering apoptosis, such as leukemias and multiple myeloma, would be more sensitive to a KSP inhibitor such as ARRY-520. Since ARRY-520 was particularly active in *in vivo* models of leukemia and myeloma, hematological malignancies are a rational setting to conduct initial proof of concept studies. The activity of ARRY-520 in taxane resistant and insensitive xenograft models supports the evaluation of ARRY-520 in patients with taxane resistant or refractory tumors. Phase 1/2 clinical trials with ARRY-520 for treatment of solid tumors and multiple myeloma are in progress, and promising early signs of biological activity have been observed in a phase 1 trial in patients with advanced or refractory leukemia (32). Two patients experienced a complete reduction in peripheral blasts, and one of these patients experienced a 70% reduction in bone marrow blasts.

Acknowledgements

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