

Mitotic Arrest in Tumors as a Pharmacodynamic Marker for Inhibition of the Mitotic Kinesin KSP by *Ispinesib*, a novel KSP inhibitor

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Abstract

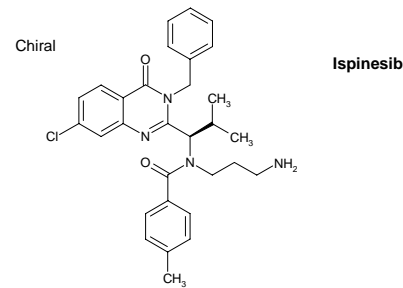
Ispinesib (SB-715992) is a novel mitotic kinesin inhibitor that is currently in phase II studies. This compound has anti-tumor activity in several xenograft models and causes mitotic arrest with monopolar spindles in cultured cells and in dividing cells *in vivo*. The ability to demonstrate an increase in mitotic cells in tissues from patients treated with *ispinesib* is an important surrogate in establishing a biologically effective dose. In this study, we explored methods for measuring the proportion of mitotic cells in tissue samples from nude mice harboring grafts of human tumors. Mice with advanced Colo205 tumors were treated with *ispinesib* at doses spanning a range previously shown to be efficacious. Twenty four hours after drug administration, tumors were harvested, dispersed into single cells, stained with propidium iodide and examined by flow cytometry for mitotic cells. Relative to placebo treated mice, tumors from drug-treated animals had a significantly greater number of cells with a 4N DNA content suggesting mitotic arrest. Tumor sections were also examined by immunohistochemical (IHC) staining with antibody against phosphorylated histone H3, a hallmark mitotic epitope, and this verified the accumulation of mitotic cells. Simultaneous staining of chromatin with DAPI further confirmed in tumor tissue the formation of DNA rosettes, a characteristic feature of monopolar spindles. Using the flow cytometry method, *ispinesib* was shown to induce dose-dependent accumulation of mitotic cells only at doses sufficient to slow tumor growth in mice. These findings suggest that analysis of patient biopsies by flow cytometry may prove useful as a fast and quantitative method for demonstrating biological activity of KSP inhibitors in clinical studies.

Introduction

Ispinesib (SB-715992) is an inhibitor of the mitotic kinesin KSP that blocks assembly of a functional mitotic spindle, thereby causing cell cycle arrest in mitosis and subsequent cell death. The mitotic spindle has long been an important functional target in cancer chemotherapy as demonstrated by the anti-tubulin agents vincristine, vinblastine and vinorelbine (Vinca alkaloids), and the taxanes paclitaxel and docetaxel. *Ispinesib* acts via a novel mechanism: inhibition of a mitotic kinesin motor protein, KSP. The expression profiles of KSP mRNA in normal human tissues are consistent with expression of KSP only in proliferating cells, as is overexpression in tumor tissue relative to normal adjacent tissue. Moreover, a correlation of KSP protein and mRNA levels has been observed in human cell lines cultured *in vitro*, including absence of expression in terminally differentiated post-mitotic neurons. In total, KSP appears to function exclusively in mitosis, and is required for centrosome separation and formation of a bipolar mitotic spindle. No role for KSP outside of mitosis has been demonstrated. As KSP is not involved in non-mitotic processes such as neuronal transport, it is expected that *ispinesib* would not cause the neuropathy often associated with the tubulin agents. *Ispinesib* has anti-tumor activity in several xenograft models and causes mitotic arrest with monopolar spindles both in cultured cells and within tumor xenografts *in vivo*. The ability to demonstrate an increase in mitotic cells in tissues from patients treated with *ispinesib* is an important surrogate in establishing a biologically effective dose. We have investigated the possibility of using analysis of tumor samples by flow cytometry as a surrogate end-point for either tumor regression or IHC for demonstrating biological activity of KSP inhibitors in xenograft studies.

Methods

Female CD-1 nu/nu mice with advanced Colo205 xenografts were used for these studies. *Ispinesib* was administered intraperitoneally in a vehicle consisting of 2% dimethylacetamide + 2% Cremophor EL + 96% acidified water [pH 5.0]. Placebo-treated animals were dosed with vehicle alone.



Efficacy studies

Ispinesib was administered at the indicated doses on a q4dx3 schedule (5 mice per group). Tumor volumes were measured at regular intervals out to 130 days post-implantation. Complete regressions (CR) were defined as tumor volume measurements ≤ 14 cu mm for three consecutive measurements. Partial regressions (PR) were defined as tumor volume measurements $\leq 50\%$ of initial tumor volume for three consecutive measurements.

IHC evaluation

Tumors were harvested at 24hrs after dosing with *ispinesib* or vehicle alone, frozen, sectioned, and immunostained for the mitotic marker phospho-Histone H3 (pHH3, red) or DNA (DAPI, blue). Tumors treated with *ispinesib* exhibited higher % pHH3 positive cells, as well as a more disorganized architecture and rosette DNA (arrows A,C), a hallmark of KSP inhibition. The mitotic index (pHH3 positive nuclei/all nuclei) was quantitated using ImagePro software (4 fields per tumor section, 2 tumors per dose) and determined for a range of *ispinesib* doses, vehicle, or paclitaxel (30mg/kg).

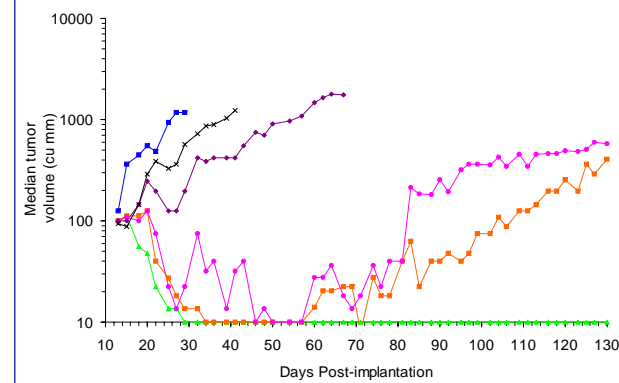
FACS analysis of solid tumors

Twenty four hours after dosing with either *ispinesib* or vehicle alone, mice were euthanized using carbon dioxide. The tumors were carefully excised and a cell suspension prepared by manually passing the tumor through a cell sieve using a total of 15 ml of PBS. The cell suspension was centrifuged at 1500 rpm (450x g) for 7 minutes. The cell pellet was then resuspended in 50ul citrate buffer in preparation for propidium iodide staining the dispersed nuclei using the Vindelov protocol. The DNA content of each sample was analysed by flow cytometry using a FACScan (Becton Dickerson). Data were plotted as number of events versus propidium iodide fluorescence intensity. From these plots, the G2M (4n DNA)/G1 (2n DNA) ratio for each sample was calculated and used as a sensitive metric for cell cycle arrest. The sub-G1 fraction was also determined. Representative histograms are shown.

All *in vivo* procedures were carried out in accordance with protocols approved by the GSK Institutional Animal Care and Use Committee

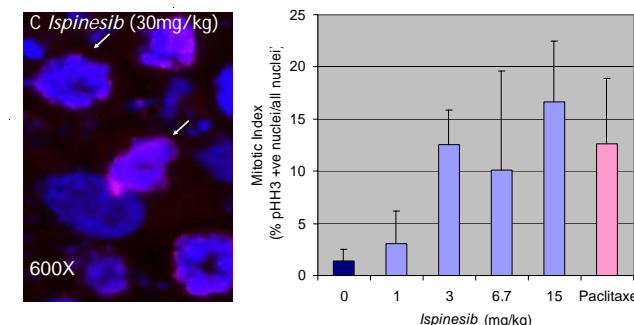
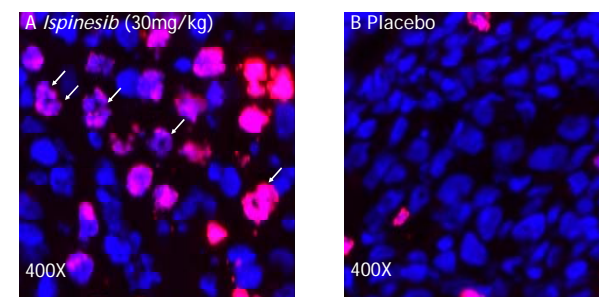
Results

Ispinesib causes complete regression of advanced sc human Colo205 colon carcinoma xenografts



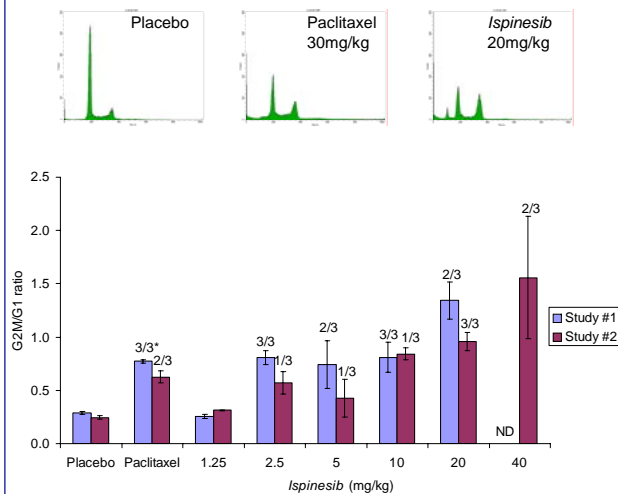
<i>Ispinesib</i> (mg/kg)	Tumor regression (complete/partial)	Tumor growth delay (T-C ₁₀₀₀ days)
40	Toxic	Toxic
20 \blacktriangle	5CR	"Cure"
10 \blacksquare	2CR/3PR	>100
5 \blacklozenge	1CR/3PR	>100
2.5 \blacklozenge	0	27
1.25 \times	0	9
Placebo \blacksquare	0	0

Ispinesib induces formation of monopolar spindles *in vivo* and a dose-dependent increase in the mitotic epitope, phospho-histone-H3



Phospho-Histone H3 (pHH3, red)
DNA (DAPI, blue)
Arrows on figures A and C donate rosette DNA, a hallmark of KSP inhibition.

Ispinesib induces dose-dependent accumulation of mitotic cells in Colo205 xenografts



*Number of samples with >30% sub G1 events/Total number of samples

Anti-tumor efficacy correlates with increase in mitotic arrest determined either by flow cytometry or IHC

Flow cytometry

	<i>Ispinesib</i> (mg/kg)					
	1.25	2.5	5	10	20	40
% of mice with tumor regression (5 studies)	0	4	48	96	100	Toxic
Median Tumor growth delay (days) (5 studies)	7	26	32	100	100	Toxic
Mitotic index (G2M/G1 Treated/G2M/G1 placebo) (2 studies)	1.1	2.6	2.2	3.1	4.3	5.8

IHC

	<i>Ispinesib</i> (mg/kg)				
	0	1	3	6.7	15
% Mitotic index (pHH3 positive cells/total cells)	0	2.5	12	10	16

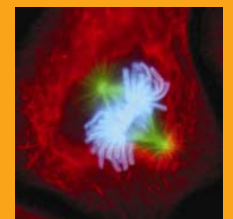
Comparison of IHC vs flow cytometry as a method of demonstrating biological activity of novel KSP inhibitors

IHC

- Good correlation between increase in pHH3 positive cells and efficacy in human tumor xenografts
- Visualization of on target effect (monopolar spindles)
- Time consuming
- Tissue sections may not be representative of tumor as a whole

Flow cytometry

- Good correlation between increase in G2M/G1 ratio and efficacy in human tumor xenografts
- Rapid
- May more accurately reflect tumor as a whole



Abstract C196

Conclusions

- *Ispinesib* induced complete regression of human Colo205 xenografts at doses down to one quarter of its MTD
- *Ispinesib* treatment of advanced Colo205 xenografts was associated with mitotic arrest and monopolar spindle formation. These observations are consistent with the mechanism of action of *ispinesib* and with cell culture findings
- Using both IHC and flow cytometry, *ispinesib* was shown to induce dose-dependent accumulation of mitotic cells only at doses sufficient to slow tumor growth in mice
- These findings suggest that analysis of patient biopsies by flow cytometry may prove useful as a fast and quantitative alternative to IHC for demonstrating biological activity of KSP inhibitors in clinical studies especially when access to tumor tissue is limited