

## Response:

### Evidence that survivin inhibits caspase-3 activity

Earlier this year, we provided a definitive report indicating that there exist at least 3 murine survivin mRNA variants, each encoding a distinct protein.<sup>1</sup> We demonstrated, with appropriate controls, that both survivin<sub>140</sub> and survivin<sub>121</sub> are able to inhibit caspase-3 activity, while survivin<sub>40</sub> does not.

In contrast to the claims of Altieri's group in their letter, several reports support our finding that survivin interferes with caspase-3 activity. These include the following: Reed's group reported that "survivin was able to substantially reduce caspase activity, as measured by cleavage of a tetrapeptide substrate, AspGluValAsp-aminofluorocoumarin. Similar results were obtained in intact cells when Survivin was overexpressed."<sup>2</sup>(p5315) Altieri's group noted in their *Nature* paper that "like other IAP proteins, survivin inhibits the terminal effectors caspase-3 and caspase-7."<sup>3</sup>(p583) Kobayashi et al demonstrated that overexpression of murine survivin in Rat1 cells inhibited caspase-induced cell death and also that a purified GST fusion protein encoding murine survivin could bind directly to caspase-3.<sup>4</sup> This group further transfected Jurkat cells with epitope-tagged survivin and showed by immunoprecipitation with anti-caspase-3 antibodies that survivin "can bind efficiently to processed caspase 3."<sup>4</sup>(p1460) In their *Nature Cell Biology* paper, Altieri's group once again noted that survivin regulates apoptosis via caspase-3: "Expression of survivin (C84A) or survivin antisense cDNA also resulted in increased activity of the apoptosis effector caspase-3, as judged by hydrolysis of the fluorogenic caspase-3 substrate."<sup>5</sup>(p461) Altieri's group also performed studies on cultured endothelial cells and reported that "[r]ecombinant expression of green fluorescent protein survivin in endothelial cells reduced caspase-3 activity and counteracted apoptosis induced by tumor necrosis factor."<sup>6</sup>(p393) And finally, a recent report confirms that the down-regulation of survivin mRNA levels by an antisense approach results in increased caspase-3 activity.<sup>7</sup>

We note that many of the data indicating that survivin interferes with caspase-3 activity predate our own and, in several cases, have actually been provided by Altieri's group. We are not in a position to assess fully the experiments described by Altieri's group and

would point out only that they are not identical to our own, in contrast to what that group claims. In addition, we would add that we have observed that the activity of recombinant survivin is highly dependent on the method chosen for its synthesis and purification. Based on our experiments and the current published data, we believe that specific forms of survivin do inhibit caspase-3 activity. Nevertheless, we agree that the means by which survivin participates in the apoptosis balance requires further investigation, and we hypothesize that the alternatively spliced isoforms of survivin may modulate apoptosis differentially.

**Edward M. Conway and Désiré Collen**

*Center for Transgene Technology and Gene Therapy  
Flanders Interuniversity Institute for Biotechnology  
University of Leuven, Leuven, Belgium*

**Andre Schuh**

*Department of Medicine and the Institute of Medical Science  
University of Toronto  
Toronto, Ontario, Canada*

### References

1. Conway EM, Pollefeyt S, Cornelissen J, et al. Three differentially expressed survivin cDNA variants encode proteins with distinct antiapoptotic functions. *Blood*. 2000;95:1435-1442.
2. Tamm I, Wang Y, Sausville E, et al. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Res*. 1998;58:5315-5320.
3. Li F, Ambrosini G, Chu EY, et al. Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature*. 1998;396:580-584.
4. Kobayashi K, Hatano M, Otaki M, Ogasawara T, Tokuhisa T. Expression of a murine homologue of the inhibitor of apoptosis protein is related to cell proliferation. *Proc Natl Acad Sci U S A*. 1999;96:1457-1462.
5. Li F, Ackermann EJ, Bennett CF, et al. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat Cell Biol*. 1999;1:461-466.
6. O'Connor DS, Schechner JS, Adida C, et al. Control of apoptosis during angiogenesis by survivin expression in endothelial cells. *Am J Pathol*. 2000;156:393-398.
7. Olie RA, Simoes-Wüst AP, Baumann B, et al. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. *Cancer Res*. 2000;60:2805-2809.

## To the editor:

### Another possible mechanism of resistance to STI571

Le Coutre et al<sup>1</sup> report the characterization of a cell line (LAMA84R) that expresses the oncogenic tyrosine kinase Bcr/Abl but is resistant to the tyrosine kinase inhibitor STI571. Those authors argue that this resistance is solely due to overexpression of the Bcr/Abl protein as a result of gene amplification.

An alternative explanation for this data is that some fraction of the Bcr/Abl in the resistant cell line has developed partial resistance to STI571 through a point mutation in the ATP binding region. To test this hypothesis, Le Coutre et al amplified a BCR/ABL fragment from both cell lines, sequenced the product, and compared it to the known nucleotide sequence. For this comparison, those authors chose a stretch of 87 nucleotides (29 amino acids<sup>1</sup>(Figure 5)) that they refer to as the "ATP binding domain" of BCR/ABL. How this stretch of sequence was chosen

is unclear, but what is certain is that it does not represent even the major fraction of the ATP binding region of BCR/ABL. The residues that *directly contact* ATP in a typical protein kinase span a region of 130 to 160 amino acids in primary sequence. Even this subset does not include many residues that do not directly contact ATP, but which when mutated could readily have structural effects that influence inhibitor sensitivity.

Resistance of Bcr/Abl to STI571 would presumably be indicated by reduced sensitivity to the drug in an in vitro kinase assay. Le Coutre et al found that both cell lines display sensitivity to STI571 but that "[d]iffering from Western blot experiments, the inhibition of kinase activity [in vitro] was never complete, even at 10  $\mu$ M [concentration of STI571]."<sup>1</sup>(p1762) This result is inconsistent with the reported potency of STI571 against the Abl kinase in vitro ( $IC_{50} = 0.038 \mu$ M)<sup>2</sup> and suggests

that some fraction of the immunoprecipitated Abl kinase activity in this study possessed reduced sensitivity to the drug.

Le Coutre et al attempt to reconcile this discrepancy in the following way: "However, it has to be considered, in the immunocomplex kinase assay, that very high, nonphysiological ATP concentrations (>20  $\mu$ M) are present, which compete with STI571 for the ATP-binding pocket of *bcr/abl*."<sup>1(p1762)</sup> This statement is not true. Physiological ATP concentrations typically range from 1 to 10 mM, which is 100-fold *higher* than le Coutre et al's reported assay conditions; as a competitive binder, STI571 should actually be more potent against the immunocomplexed kinase than *in vivo*.<sup>3</sup>

A thorough characterization of mechanisms of cellular resistance to STI571 would provide valuable insight into the potential utility of competitive kinase inhibitors as therapeutics. The present study, however, presents incomplete evidence that cannot be

consistently explained by the hypothesis that resistance is solely due to gene amplification.

#### Zachary A. Knight

Department of Cellular and Molecular Pharmacology  
University of California, San Francisco  
San Francisco, CA

#### References

1. Le Coutre P, Tassi E, Varella-Garcia M, et al. Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. *Blood*. 2000;95:1758-1766.
2. Buchdunger E, Zimmermann J, Mett H, et al. Inhibition of the abl protein-tyrosine kinase *in vitro* and *in vivo* by a 2-phenylaminopyrimidine derivative. *Cancer Res*. 1996;56:100-104.
3. Cohen P. The development and therapeutic potential of protein kinase inhibitors. *Curr Opin Chem Biol*. 1999;3:459-465.

## Response:

### Gene amplification the most likely mechanism of resistance to STI571 in LAMA84R cells

In our recent publication (le Coutre et al<sup>1</sup>), we presented the generation and analysis of a cell line positive for BCR/ABL but resistant to the tyrosine kinase inhibitor STI571. The cell line generated in our laboratory (LAMA84R) exhibited a 10-fold decrease in sensitivity to STI571, which normally induces apoptosis in sensitive parental LAMA84 cells.<sup>2</sup> Our results indicated that the induction of resistance in LAMA84R is likely to be caused by the amplification of the BCR/ABL gene leading to increased expression of the oncogenic fusion protein in resistant cells. Our conclusion was based on the observation that the transcription and expression of *bcr/abl* is increased in LAMA84R compared to parental cells as shown by Northern and Western blot analyses. Using fluorescence *in situ* hybridization (FISH) analysis, we demonstrated that the alteration of *bcr/abl* expression was accompanied by gene amplification of *BCR/ABL* in cells resistant to STI571 resulting in an approximately 4-fold increase in *bcr/abl* expression.

To investigate the possibility of resistance to STI571 caused by a point mutation within the *BCR/ABL* gene that could result in a reduction of binding affinity to STI571, we have performed sequence analysis of the ATP binding region located within the Abl portion of the fusion protein. STI571 binds to *bcr/abl* as a competitor of ATP.<sup>3</sup> The complete tyrosine kinase domain of cAbl spans amino acids 235-486 and includes the putative ATP binding lobe composed of the conserved amino acids 248-256 and 271, respectively.<sup>4</sup> These amino acids are encoded by the corresponding nucleotides 744-813 of the cAbl coding sequence (GenBank accession number M14725; see also SwissProt accession number P00519). The nucleotide sequence of the ATP binding domain and approximate flanking sequences derived from LAMA84R appeared to be identical to the published one. But the possibility that single copies of the amplified *BCR/ABL* genes contain mutations that render the resulting protein less susceptible to STI571 cannot be entirely excluded even by sequencing the entire gene, as proposed by Dr Knight. LAMA84R contain approximately 14 copies of the *BCR/ABL* gene. Because reverse transcription and subsequent sequencing of the resulting PCR product have to be employed, the exact copy of the *BCR/ABL* gene being analyzed cannot be determined. It is therefore possible that a *BCR/ABL* gene not presenting the putative mutations be sequenced, with false negative results.

Following our publication,<sup>1</sup> 2 additional reports (Mahon et al<sup>5</sup> and Griffin and Weisberg<sup>6</sup>) examined the development of resistance to STI571 *in vitro*. In these reports upregulation of *bcr/abl* expression either transiently or by gene amplification were suggested to constitute major mechanisms by which resistant cells overcome the apoptotic effect of STI571. Both groups performed PCR analysis of the *BCR/ABL* gene. Whereas one group analyzed the nucleotide sequence encoding the entire kinase domain,<sup>5</sup> the second group performed sequence analysis of a putative ATP binding region that was smaller than the one analyzed in our report.<sup>6</sup> But in neither publication was the detection of a mutation within the ATP binding region nor kinase domain reported. In addition, one group<sup>6</sup> also probed the *BCR/ABL* gene by Southern blot assay, employing several restriction enzymes to look for mutations, with negative results.

The mechanism of resistance to STI571 proposed in our publication is supported by our finding that LAMA84R cells remain susceptible to higher doses of the drug. This was demonstrated by dephosphorylation of *bcr/abl* detected by antiphosphotyrosine Western blotting and the reduction of kinase activity measured by substrate phosphorylation of GST-CH1-Shc *in vitro*. In both assays, we observed a parallel reduction of *bcr/abl* kinase activity in both LAMA84R and nonresistant parental cells.

As Dr Knight correctly points out, physiological ATP concentrations are approximately 100-fold higher than the 20  $\mu$ M used in immunoprecipitation kinase assay. But whereas the ATP pool inside cells is competed for by many different enzymes, in the kinase assay it is at the complete disposal of *bcr/abl*. In addition, it must be remembered that the levels of phosphorylation inside cells (as evaluated by Western blot assay) reflect the balance between phosphorylation and dephosphorylation, whereas in the kinase assay no dephosphorylation is possible because phosphatases are not assumed to be present and phosphatase inhibitors are added in the reaction buffer. For all these reasons, as we pointed out, neither of the 2 assays used to analyze the STI571-sensitivity of *bcr/abl* derived from LAMA84R and parental cells allow the exact quantification of kinase activity but are designed to compare *bcr/abl* derived from resistant and susceptible cell lines. Band intensity between the 2 assays therefore cannot be compared directly.

Our study provides first evidence that resistance to STI571 can

be caused by the amplification of the *BCR/ABL* gene in vitro. Although it is not completely possible to rule out mutations, we think the most likely explanation for the observed resistance in this case resides in the amplification of the *BCR/ABL* gene. Other publications that report the generation of similar cell lines and analyze mechanisms of resistance to STI571 complement our original results.

**Carlo Gambacorti-Passerini, Philipp le Coutre, Elena Tassi, and Holger Ruchatz**

Department of Experimental Oncology  
Istituto Nazionale Tumori  
Milan, Italy

## References

1. Le Coutre P, Tassi E, Varella-Garcia M, et al. Induction of resistance to the

- Abelson inhibitor STI571 in human leukemic cells through gene amplification. *Blood*. 2000;95:1758-1766.
2. Gambacorti-Passerini C, le Coutre P, Mologni L, et al. Inhibition of the Abl kinase activity blocks the proliferation of BCR/ABL+ leukemic cells and induces apoptosis. *Blood Cells Mos Dis*. 1997;23:380-394.
3. Buchdunger E, Zimmerman J, Mett H, et al. Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. *Cancer Res*. 1996;56:100-104.
4. Fainstein E, Einat M, Gokkel E, et al. Nucleotide sequence analysis of human *abl* and *bcr-abl* cDNAs. *Oncogene* 1989;4:1477-1481.
5. Mahon FX, Deiniger M, Schultheis B, et al. Selection and characterization of *BCR-ABL* positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanism of resistance. *Blood*. 2000;96:1070-1079.
6. Weisberg E, Griffin JD. Mechanism of resistance to the ABL tyrosine kinase inhibitor STI571 in BCR/ABL-transformed hematopoietic cell lines. *Blood*. 2000;95:3498-3505.

## To the editor:

### Prevalence of pyruvate kinase deficiency in a northern European population in the north of England

Attention has been recently drawn to the difficulty in establishing the actual prevalence of pyruvate kinase (PK) deficiency in the general white population. Beutler and Gelbart<sup>1</sup> have estimated this to be 51 cases per million white population by gene frequency studies. We have been centrally registering all patients with PK deficiency within the former Northern Health Region of the United Kingdom since 1974. This is a mainly static population of 3.1 million<sup>2</sup> with less than 3% from ethnic minority groups and a minimal apparent incidence of inbreeding.

During the period since 1974, we have had 10 patients with PK deficiency registered (see Table 1). The diagnoses were based on pyruvate kinase activity measurement. No genetic diagnoses were made. Our observed prevalence of 3.3 per million in a mainly white population (more than 97% white) is more than an order of

magnitude lower than the 51 per million predicted by Beutler and Gelbart using gene frequency techniques/methods.

During the decade 1960-69 (secondary "baby boomers" peak in the United Kingdom), peak prevalence was achieved at a rate of 8.5 per million of population. From our registrations there are very few older patients. We postulate that a possible explanation of a low prevalence of patients more than 60 years old is that the advent of routine blood transfusion and neonatal exchange transfusion did not occur until the post-World War II period. One further potential explanation of the discrepancy between observed and predicted prevalence of PK deficiency is that the condition is associated with a significant intrauterine death rate.

**Table 1. Prevalence of PK in Northern Health Region, United Kingdom, 1930-99**

Decade of birth	Population in age group	Number of patients with PK	Prevalence per million
1990-99	NA	0	—
1980-89	400 000	1	2.5
1970-99	390 000	2	5.0
1960-69	470 000	4*	8.5
1950-59	430 000	1	2.5
1940-49	403 000	1	2.5
1930-39	330 000	1	†
Total population	3 100 000	10	3.2

NA, not available.

\*Includes one pair of siblings.

†See text for explanation.

**Peter J. Carey, John Chandler, Alex Hendrick, Michael M. Reid, Peter William G. Saunders, Hazel Tinogate, Penelope R. Taylor, and Nicholas West, on behalf of the Northern Region Haematologists Group**

Department of Haematology  
Royal Victoria Infirmary  
Newcastle upon Tyne  
England

## References

1. Beutler E, Gelbart T. Estimating the prevalence of pyruvate kinase deficiency from gene frequency in the general white population. *Blood*. 2000;95:3585-3588.
2. Office of Population and Census Studies (OPCS). Population projections by the government actuary, United Kingdom: mid-1987-based projection. Her Majesty's Stationery Office, London, United Kingdom, 1991.

## Response:

### PK deficiency prevalence and the limitations of a population-based survey

There are no other studies in which an attempt has been made to relate the prevalence of pyruvate kinase (PK) deficiency to a population base, and therefore Carey et al's studies are of

interest. It must be pointed out, however, that disease prevalence estimates based on the detection of affected patients are bound to be substantially lower values than estimates based on the