SUPPORTING ONLINE MATERIAL

ONLINE METHODS

Mice. CD1 and C57BL/6 mice were housed in specific pathogen free conditions in The Walter and Eliza Hall Institute (WEHI) mouse breeding facility (Parkville, VIC, Australia) under controlled conditions of 12 h light: 12 h dark, with free access to water and mouse chow. All animal procedures were approved by the WEHI Animal Ethics Committee and were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, including breeding commencing from the age of six weeks (PN42). CD1 mice were treated at PN5 with vehicle or imatinib, (Novartis), 7.5 mg/kg i.p. or cisplatin, (Mayne Pharma, hospital grade), 5 mg/kg, or both imatinib and cisplatin administered together as reported in Gonfloni et al[1]. In some experiments, C57BL/6 mice at PN5 or PN7 received a single dose of imatinib (7.5 mg/kg i.p.) or vehicle (mouse tonicity PBS), followed 2 h later by cisplatin (5 mg/kg i.p.) or vehicle. The reverse combination was also performed (cisplatin administered 2 h prior to imatinib). Biological activity of imatinib was verified in a BCR-ABL$^+$ leukemic cell line as previously reported[2]. K562 human Ph1$^+$ (BCR-ABL$^+$) leukemia-derived cells were cultured and treated with 0.156-10.0 μM imatinib for 72 h, with previously reported loss of cell viability observed, as determined by the Cell Titre Glo assay **(Data not shown)**. Mice were killed and ovaries harvested at PN10 or observed until 6 weeks (CD1 strain) or 7 weeks (C57BL/6 strain) of age when breeding trials commenced.

In vitro **whole organ culture of ovaries.** Ovaries were harvested from PN5 C57BL/6 females and cultured on floating filters in MEM/Ham's-F12 supplemented with 0.01%

BSA, 0.01% Albumax II, 0.05 mg/mL L-ascorbic acid, 5X ITS-X, 75 μg/mL penicillin-G and 50 μg/mL streptomycin sulfate (all supplied by Gibco), at 37 °C in 5% CO_2. Ovaries were cultured for 2 h in the presence of imatinib (10 μM) or vehicle (mouse tonicity PBS) before being treated with cisplatin (20 μM) or vehicle. Ovaries were collected for analysis after a further 24 or 48 h of incubation. Imatinib was dissolved in DMSO. Cisplatin was diluted in saline. Imatinib and cisplatin doses were selected on the basis of a previously published study[1].

Follicle quantification. Quantification of numbers of follicles (which contain oocytes), were categorized as primordial, primary or secondary follicles, (mean ± SEM) and were expressed per 10^4 μm^2 (or per 10^6 μm^2 for adults) ovarian tissue area[3,4]. Randomly selected left or right ovaries from cisplatin-treated, imatinib-treated, imatinib plus cisplatin treated or vehicle-treated mice were fixed for 2 h in Bouin's fixative or 4% PFA, processed into paraffin and 5 μm serial sections of each ovary were stained with hematoxylin and eosin. From each set of serial sections, 4-5 sections from equal intervals through whole ovaries (to avoid counting follicles twice) were selected for semi-quantitative estimation of follicles using morphological criteria previously described[3,4]. Follicles with a morphologically normal oocyte nucleus were counted in sections and section areas (in μm^2) were measured with image analysis software (Infinity Analyzer v5, Lumenera, Ottawa, Canada). The total number of follicles counted from *in vivo* experiments at PN10 were 1800 and 1802 (Primordial follicles from CD1 and C57BL/6 mice respectively), 239 and 600 (Primary follicles) and 489 and 370 (Secondary follicles) (Refer also to **Supplementary Table 1**); and from *in vivo* experiments at PN49 were 321

(Primordial follicles), 107 (Primary follicles) and 117 (Secondary follicles) (Refer also to **Supplementary Table 2**) and *in vitro* experiments were 4297 (Primordial follicles), 1161 (Primary follicles) and 51 (Secondary follicles) (Refer also to **Supplementary Table 4**). Pyknotic oocytes were characterized by densely stained compacted nuclear chromatin as single or several clumps and intense eosinophilic cytoplasm. "Pyknotic" was conservatively considered as classic condensed structure enclosed by follicular cells or a severely dysmorphic/dense nucleus not typical of a healthy oocyte. At PN10, the numbers of pyknotic cells were low, as presumably most dead oocytes had been cleared within the five days between treatment and analysis. When the number of pyknotic bodies per ovary section exceeded 100, quantitative data were expressed as $> 5/10^4$ μm^2 tissue area.

Fertility trials. CD1 strain: female mice treated as described above at PN7 with vehicle or imatinib alone or cisplatin alone or both imatinib plus cisplatin administered together were allowed to mature. For each treatment cohort, 3 mice were harvested at PN42 for histologic analysis. The remainder commenced breeding trials at PN42 with proven wt (CD1) males as described in Gonfloni et al[1]. Gonfloni et al[1] defined infertility as failure to plug within one week of mating: in their study, pups were left with the mother for one week and following sacrifice of the pups aged PN7, the mother was again mated one week later i.e. "the mating procedure was repeated at regular intervals (about every 5 weeks)".

C57BL/6 strain: female mice at PN7 received a single dose of imatinib (7.5 mg/kg i.p.) or vehicle (mouse tonicity PBS), followed 2 h later by cisplatin (5 mg/kg i.p.) or vehicle. In

the mouse, folliculogenesis from primordial follicle activation to ovulation requires about 20 days[5]. Through 45 days, all growing follicles (primary and more mature) present in the PN5-7 ovaries at the time of treatment with cisplatin, would have either undergone atresia or completed folliculogenesis and ovulated prior to the initiation of fertility trials commenced at PN49. Ovulatory follicles present at or beyond 7 weeks following treatment were therefore considered to be derived from primordial follicles present at PN5 or PN7 that had survived cisplatin treatment, which indeed is not sterilizing until after 3 rounds of breeding[1]. Litters were inspected at 0800 on the day of birth, then twice weekly and at weaning. Following delivery of a litter, the breeding pair remained together to allow subsequent mating and pregnancy to occur without disruption. The outcomes of six rounds of breeding/litters were recorded. The proportions of females in each treatment group noted to be infertile at each breeding round (litter #1- #6) were calculated (failure to become pregnant despite being caged with a proven male breeder, when observed for 12 weeks post previous delivery). The total numbers of females (vehicle treatment = 22; imatinib treatment = 29; cisplatin treatment = 36; imatinib+cisplatin treatment = 31), litters (vehicle treatment = 84; imatinib treatment = 121; cisplatin treatment = 113; imatinib+cisplatin treatment = 112) and pups (vehicle treatment = 615; imatinib treatment = 825; cisplatin treatment = 641; imatinib+cisplatin treatment =682) were included.

Details of how the proportions of fertile females and average litter size were defined, calculated and statistically analyzed were not provided by Gonfloni et al[1]. We describe

here the considerations for our C57BL/6 breeding analyses (summary in Supplementary Table 4):

i) All females receiving treatments described above, matured to PN49 and mated with proven wt (C57BL/6) male breeders, were included in the breeding analyses. Breeding females were noted to be pregnant including close to term, to deliver a litter (or part of a litter – see below) or to fail to become pregnant despite being caged with a proven male breeder, when observed for 12 weeks post previous delivery (infertile) at each breeding round. Mating pairs were not separated, in order to facilitate subsequent pregnancies. Infertility was censored in a Kaplan-Meier analysis. Breeders sacrificed for birthing difficulties (vehicle treatment = 3; imatinib treatment = 5; cisplatin treatment = 5; imatinib+cisplatin treatment = 4) or dying post delivery (one mouse, vehicle group) were included in the analysis until their sacrifice/death.

ii) The number of pups born was documented for each litter. If litters contained evidence of maternal cannibalism of pups, the number of pups was deemed to be inaccurate for that litter and the litter was excluded from the average pup/litter analysis (affecting all cohorts to a similar extent: number of litters excluded (vehicle treatment = 6 out of 93 litters; imatinib treatment = 12 out of 135 litters; cisplatin treatment = 12 out of 128 litters; imatinib+cisplatin treatment = 12 out of 122 litters)).

iii) If a breeding female was documented to be pregnant at late term and was found in birthing difficulties, delivering no live pups, or was found (at 0800 am) to be no longer pregnant, without evidence of pups (“0” pups), with the mother deemed to

have cannibalized the litter, the litter was excluded from the average pup/litter analysis for reasons of inaccuracy (affecting all cohorts to a similar extent: number of litters excluded (vehicle treatment = 3 out of 93 litters; imatinib treatment = 2 out of 135 litters; cisplatin treatment = 3 out of 128 litters; imatinib+cisplatin treatment = 2 out of 122 litters)).

iv) The numbers of mice that had not yet completed six litters at the time of writing (affecting all cohorts to a similar extent): vehicle treatment = 11 out of 22 female breeders; imatinib treatment = 13 out of 29; cisplatin treatment = 18 out of 36; imatinib+cisplatin treatment = 15 out of 31. These breeders were less than 12 weeks following delivery of their most recent litter and were therefore considered to be fertile at the time of writing.

v) Upon failure of a breeding female to become pregnant at any breeding round (defined as 12 weeks from the date of last delivery), the mouse was deemed to be infertile, sacrificed and both ovaries harvested for histologic analysis (Supplementary Fig. 4C).

Statistical analysis. Follicle quantification data are presented as means ± SEM and statistical analysis of follicle numbers was performed using GraphPad Prism software (GraphPad Software Inc, version 5.0a La Jolla, CA, USA). Data were analyzed by one-way ANOVA and the significance determined by the Tukey's *post hoc* multiple comparison test. Prism software was also used for generating Kaplan-Meier plots and for performing statistical analysis (using a log rank test) of likelihood of infertility.

For further analysis of C57BL/6 fertility data, we tested the null hypothesis that females in the cisplatin alone (*cis)* and the cisplatin+imatinib (*cis+ima*) treatment groups had the *same* mean numbers of pups for all of litters *1* to *6.* The test made use of data on the numbers of pups born to fertile females in the two groups at each of litters *1* to *6*, but instead of carrying out six separate two-sample tests, one for each litter, we computed the six two-sample *t*-statistics (unequal variance) and *combined* them into a single, summary test statistic. The main reason for doing this was the fact that the six *t*-statistics are not independent, because females in each group contribute to several of the *t*-statistics: there are *repeated measures* on females. This makes the interpretation of the litter-by-litter *t*-statistics difficult, whereas it becomes straightforward with the use of a summary statistic. We utilized two principal summary statistics: the sum *S* of the absolute values and the largest *L* of the six *t*-statistics. For good measure, we also looked at the sum of squares *SS* of the six *t*-statistics, and the total *T* and average number *A* of pups per mother. Each of these statistical tests can be used to test the null hypothesis, and each will have power against different alternative hypotheses. In no case was the *p*-value associated with any of these summary test statistics smaller than *0.2*; i.e. there was no evidence of differences between treatment groups that would be significant at any conventional level.

The *p*-values were calculated in a manner that respected the repeated measure feature of the data, as well as embodying the null hypothesis. Females from the two treatment groups were pooled, and a random sample from the pooled females of size equal to that of the *cis* group was taken, and designated *cis**. The remaining females were then

designated *cisplatin+imatinib**. The six *t*-statistics and the summaries *S*, L*, SS*, T* and A** were then calculated for these random treatment groups, and recorded. This process of random sampling and calculating of summary statistics was repeated 10,000 times, leading to 10,000 sets of summary statistics under conditions reflecting the null hypothesis of no differences between the mean litter sizes of the treatment groups. The values of the summary statistics for the actual data were then viewed in relation to the 10,000 values obtained for the random assignments and *p*-values assigned to each by counting the number of random results leading to summary test statistics exceeding those observed with the actual data. The results were as follows: for the summary *S, p>0.6*; for *M, p>0.8*; *for SS, p>0.7*; for *T, p>0.4*; and for *A, p>0.3*. This procedure is termed a permutation test and we refer to Chapter 18, Section 6 of Moore *et al* [6] for a similar example.

References

1. Gonfloni, S., et al. Inhibition of the c-Abl-TAp63 pathway protects mouse oocytes from chemotherapy-induced death. Nat Med 15, 1179-1185 (2009).
2. Kuroda, J., et al. Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. Proc Natl Acad Sci U S A 103, 14907-14912 (2006).
3. Kerr, J.B., et al. Quantification of healthy follicles in the neonatal and adult mouse ovary: evidence for maintenance of primordial follicle supply. Reproduction 132, 95-109 (2006).
4. Myers, M., Britt, K.L., Wreford, N.G., Ebling, F.J. & Kerr, J.B. Methods for quantifying follicular numbers within the mouse ovary. Reproduction 127, 569-580 (2004).
5. Pedersen, T. Follicle kinetics in the ovary of the cyclic mouse. Acta Endocrinol (Copenh) 64, 304-323 (1970).
6. Moore, D.S., McCabe, G., Duckworth, W. & Sclove, S. The Practice of Business Statistics: Using Data for Decisions, (W.H. Freeman, New York, N.Y>, 2003).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1

Pre-treatment with imatinib did not protect primordial follicle oocytes from DNA damage induced death. (**A**) Quantification of primordial follicles in C57BL/6 mice that were pre-treated at PN5 with imatinib (7.5 mg/kg i.p.) or vehicle and then 2 h later exposed to cisplatin, (5 mg/kg i.p.) or vehicle and analyzed at PN10. No differences in primary and secondary follicle numbers were observed among groups (not shown). **(B)** Hematoxylin and eosin staining of ovaries harvested at PN10 and treated as in (A). Vehicle-treated and imatinib-treated ovaries show abundant primordial follicles. Marked depletion of primordial follicles (arrows) in cisplatin and imatinib+cisplatin-treated ovaries. Arrowheads indicate pyknotic bodies. Scale bar indicates 50 μm (B). **(C)**. Ovaries were harvested from C57BL/6 female pups at PN10, following treatment as in (A). Quantification (means ± SEM) of pyknotic bodies was performed at PN10. Pyknotic oocytes showed densely stained compacted nuclear chromatin as single or several clumps and intense eosinophilic cytoplasm. For comparison with vehicle-treated controls: *$p<0.05$, **$p<0.01$, ***$p<0.001$. N=4-6 ovaries per treatment group.

Supplementary Figure 2

Morphological appearance of ovaries and TUNEL staining following combined *in vitro* treatment with imatinib and cisplatin for 24 h (A) and 48 h (B). Ovaries were harvested from C57BL/6 PN5 female pups, maintained in whole organ culture and treated with imatinib (10 μM) or vehicle for 2 h, then 2 h later with cisplatin (20 μM) or vehicle. MSY2 staining was used to visualize oocytes and GCNA staining was used to

confirm the identity of primordial follicles. Primordial follicles were not detected and, instead, large numbers of pyknotic cells were observed in ovaries treated for 48 h in culture with cisplatin alone or the combination of cisplatin plus imatinib. TUNEL staining revealed increased TUNEL-positive (i.e. apoptotic) primordial oocytes following treatment with cisplatin, with or without imatinib pre-treatment at 24 h (data also included in **Supplementary Fig. 1B**). Only limited TUNEL staining was observed at 48 h post-treatment, most likely because by that time the dying oocytes were too degraded and/or had been removed by phagocytes. Scale bars indicate: 50 μm for GCNA and TUNEL and 200 μm for MSY2.

Supplementary Figure 3

Quantification of follicles at PN49 in C57BL/6 mice following combined *in vivo* treatment with imatinib plus cisplatin at PN5. C57BL/6 female pups were pre-treated at PN5 with imatinib (7.5 mg/kg i.p.) or vehicle (PBS) for 2 h and then treated with cisplatin (5 mg/kg) or vehicle and allowed to mature to PN49, whereupon mice were harvested for histologic analysis of ovaries. Quantification of follicles was performed as described in Methods. Total healthy primordial, primary or secondary follicles, (each of which contain an oocyte) were counted and the mean ± SEM expressed per 10^6 μm^2 ovarian tissue area. For comparison with vehicle-treated controls: n=3 ovaries per treatment group * $p<0.05$, ** $p<0.01$ vehicle-treated vs treated.

Supplementary Figure 4

Appearance of ovaries following combined *in vivo* treatment of C57BL/6 females with imatinib plus cisplatin at PN5 harvested at PN49 (A), at 9-11 months of age (B) or upon cessation of fertility (C). C57BL/6 female pups were pre-treated at PN5 or PN7 with imatinib (7.5 mg/kg i.p.) or vehicle (PBS) for 2 h and then treated with cisplatin (5 mg/kg) or vehicle and allowed to mature to PN49 **(A)** or 9-11 months (completing at least 6 breeding rounds) **(B)**, whereupon mice were harvested for histologic analysis of ovaries. Hematoxylin and eosin staining of ovarian sections: PN49: primordial, primary and secondary follicles were noted in vehicle- and imatinib-treated ovaries; in cisplatin and imatinib+cisplatin-treated ovaries primordial follicles were rare but empty follicles were abundant; 9-11 months: occasional primordial or primary follicles in vehicle- and imatinib-treated ovaries; empty follicles in imatinib, cisplatin, and imatinib+cisplatin-treated ovaries. Upon cessation of fertility (defined as failure to become pregnant by twelve weeks following the date of delivery of last litter), ovaries were harvested for histologic analysis, which confirmed the absence of viable ovarian follicles **(C)**. Ovaries from failed breeders showed only luteinized tissue. Primordial (arrow), primary (chevron), secondary (star) and empty follicles (arrowhead) are indicated. Scale bar represents 50 μm.

Supplementary Figure 5

Pre-treatment with imatinib did not protect primordial follicle oocytes from DNA damage induced death. (A) Ovaries were harvested from C57BL/6 mice at PN5, maintained in whole organ culture, pre-treated with imatinib (10 μM) or vehicle for 2 h

and then treated with cisplatin (20 μM) or vehicle. Quantification of follicles (means ± SEM) was performed after 24 and 48 h. Primordial follicle numbers are shown. No differences in primary and secondary follicle numbers were observed among groups (not shown). **(B)** TUNEL staining of ovaries cultured as in (A) revealed increased TUNEL-positive primordial follicle oocytes following treatment with cisplatin, with or without imatinib pre-treatment at 24 h. **(C)** Ovaries were harvested from C57BL/6 female pups at PN5, maintained in whole organ culture and treated as in (A). Quantification (means ± SEM) of pyknotic bodies was performed at 24 h and 48 h. Pyknotic oocytes showed densely stained compacted nuclear chromatin as single or several clumps and intense eosinophilic cytoplasm. For comparison with vehicle-treated controls: *$p<0.05$, **$p<0.01$, ***$p<0.001$. N=3-9 ovaries per treatment group.

Supplementary Figure 6

Pre-treatment with imatinib did not rescue cisplatin-induced loss of fertility in C57BL/6 female mice. C57BL/6 female pups were pre-treated with imatinib (7.5 mg/kg i.p.) or vehicle and then 2 h later exposed to cisplatin, (5 mg/kg i.p.) or vehicle at PN5 or PN7 and were used in breeding studies from the age of 7 weeks. **(A)** Prior treatment with imatinib did not impact on cisplatin-induced effects on litter size. The average number of pups in each litter from pregnant mice was determined (cisplatin vs imatinib+cisplatin $p>0.6$). **(B)** The likelihood of infertility in each treatment group (Kaplan-Meier analysis, cisplatin vs imatinib+cisplatin $p>0.3$; PBS curve is identical to the imatinib curve and lies behind it). 1-6 = Breeding round #1-6.