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Host Thiopurine Methyltransferase Status Affects Mercaptopurine Antileukemic Effectiveness in a Murine Model

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Abstract

Thiopurines are used for many cancers, including acute lymphoblastic leukemia (ALL). Patients with an inherited host defect in thiopurine methyltransferase (TPMT) are at high risk for life-threatening toxicity if treated with conventional dosages, but the impact on antileukemic efficacy is less clear. Herein, we treated thiopurine-sensitive BCR-ABL+ *Arf*-null *Tpmt*+/+ ALL in *Tpmt* +/+, +/−, or −/− recipient mice to test the impact of the host polymorphism on antileukemic efficacy. Median survival was similar in untreated mice of different *Tpmt* genotypes (16–18 days). However, in mice treated with low-dose mercaptopurine (such as tolerated by *TPMT*−/− patients), the difference in 30-day leukemia-free survival by *Tpmt* genotype was profound: 5% (± 9%) for *Tpmt*+/+ mice, 47% (± 26%) for *Tpmt*+/− mice, and 85% (± 14%) for *Tpmt*−/− mice ($p = 5\times10^{-8}$), indicating a substantial impact of host *Tpmt* status on thiopurine effectiveness. Among *Tpmt*+/+ recipient mice, leukemia-free survival improved with higher doses of mercaptopurine (similar to doses tolerated by wild-type patients) compared to lower doses, and at higher doses was comparable (p =0.6) to the survival of *Tpmt*−/− mice treated with the lower dose. These findings support the notion that germline polymorphisms in *Tpmt* affect not only host tissue toxicity, but also antitumor effectiveness.

Keywords

TPMT; thiopurine; acute lymphoblastic leukemia; mercaptopurine; murine leukemia model; BCR-ABL

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Conflict of Interest statement. "St. Jude allocates a portion of the income it receives from licensing inventions and tangible research materials to those researchers responsible for creating this intellectual property. Under this policy, Drs. Evans and Relling receive a portion of the income St. Jude receives from licensing patent rights related to TPMT polymorphisms."

Introduction

Acute lymphoblastic leukemia (ALL) is the most common type of cancer in children. The treatment regimen consists of several chemotherapeutics, including the thiopurine 6-mercaptopurine (6-MP or purinethol). Mercaptopurine is an anti-cancer and immunosuppressive drug also used to treat non-Hodgkin's lymphoma, Crohn's disease, and polycythemia vera.

Thiopurine methyltransferase (TPMT) methylates the thiopurines mercaptopurine and thioguanine, diverting the parent drug to the active metabolites of thioguanine nucleotides (TGN). The consequence of methylation by TPMT is complicated by the fact that the methylated secondary metabolites may enhance antileukemic and immunosuppressive effects because of their direct inhibition of purine de novo synthesis[1, 2]. TPMT activity is inherited as a Mendelian trait, with common *TPMT* coding polymorphisms resulting in the classification of patients as homozygous deficient, heterozygous, or homozygous wild type for *TPMT* genetic status. When routine doses of thiopurines are given to homozygous deficient patients, they have high levels of TGN and develop severe myelosuppression, which can be fatal[3]. The Clinical Pharmacogenetics Implementation Consortium recommends that patients with two nonfunctional *TPMT* alleles receive 10% or less of the thiopurine dose administered to patients with two functional *TPMT* alleles[4]. They also recommend starting therapy with thiopurines at 30–70% of the full dose in heterozygous patients, with monitoring of myelosuppression for further dose adjustments.

The murine model of *Tpmt* deficiency mimics the clinical toxicity and pharmacokinetics of thiopurines very well[5], with higher levels of TGN metabolites and more severe toxicity in *Tpmt* knockouts (−/−) versus heterozygous (+/−) or wild-type (+/+) mice. However, the impact of homozygous deficiency or heterozygosity of *Tpmt* on the desired antileukemic efficacy of thiopurines is not known. Herein, we used this model system to evaluate antileukemic efficacy of mercaptopurine in *Tpmt* −/−, +/−, and +/+ mice. Given at a fixed low dose that is clinically relevant for *TPMT* −/− patients, mercaptopurine significantly improved survival in *Tpmt* −/− mice compared to untreated *Tpmt* −/− mice and to mercaptopurine-treated *Tpmt* +/− and +/+ mice. With higher doses of mercaptopurine, similar to what *TPMT* +/− or +/+ patients would tolerate, leukemia-free survival increased to levels similar to *Tpmt* −/− mice treated with the low dose.

Materials & Methods

Mice

Tpmt-deficient mice were generated[5] and backcrossed to the 129×1/SvJ background (Jackson Labs, stock number 000691, Bar Harbor, Maine) to create mice that were wild-type (+/+), heterozygous (+/−) or deficient (−/−) with respect to *Tpmt*. *Arf*-null mice on a C57Bl/6×129 mixed background[6] were backcrossed onto the 129×1/SvJ background for seven generations. For purposes of generating isogenic B-lineage ALL (see below), the two strains were crossed to generate mice that were wild-type with respect to *Tpmt* and null for *Arf* to provide bone marrow for generation of transplantable ALL. All recipient mice were wild-type with respect to *Arf*. Mice were bred in-house and maintained under specific pathogen-

free conditions[7]. All experiments were conducted under the supervision of the facility's Institutional Animal Care and Use Committee (IACUC) according to an IACUC-approved protocol and requisite NIH guidelines.

Chemicals

Mercaptopurine (6-MP) was purchased from Sigma Aldrich, St. Louis, MO (Stock #M6875). Methotrexate was purchased from Parenta Pharmaceuticals, Inc (Yardley, PA).

Trimethoprim/sulfamethoxazole (Septra pediatric suspension) was purchased from Hi-Tech Pharmacal (Amityville, NY). A folic-acid-deficient purified diet was purchased from Purina TestDiet (Richmond, IN).

Tpmt activity assay

To confirm Tpmt phenotype, in terminal procedures, 1 mL blood was collected via cardiac puncture into tubes containing sodium heparin. Erythrocyte lysates were prepared and analyzed for Tpmt activity by a nonchelated radiochemical assay as previously described[5]. The assay is based on the conversion of mercaptopurine to radioactively labeled methylmercaptopurine with S-[methyl-14C]-adenosyl-L-methionine as the methyl donor.

Induction of ALL

MSCV vectors coexpressing human BCR-ABL (p185) and luciferase[8] were packaged into replication-incompetent ecotropic virions as described[9]. Marrow extracted from the long bones of 129×1SvJ *Arf*-null mice (*Tpmt* +/+) was suspended in vector-containing supernatant and plated to derive pro/pre-B cells on autologous stroma[10, 11]. Cells were maintained at 37°C (8% CO_2) in RPMI with 10% fetal calf serum (Thermo Scientific Hyclone, Logan, UT), 4 mM L-glutamine and 55 uM 2-mercaptoethanol (Gibco, Grand Island, NY). After 7–8 days of culture on autologous stroma, pro/pre-B cells were transferred to stromal-free culture for 2–3 days before cryopreservation in 90% FBS with 10% DMSO (Sigma Aldrich, St. Louis, MO) and storage in liquid nitrogen. Prior to injection, cells were thawed and recovered in culture for at least 48 hours. 2,000 *Arf*-null pro/pre-B cells bearing the p185 BCR-ABL fusion protein (confirmed to be co-expressing luciferase) were injected intravenously via tail vein into cohorts of healthy, unconditioned 8 – 10 week old male and female 129×1SvJ mice that were −/−, −/+, or +/+ with respect to *Tpmt* and +/+ with respect to *Arf*. Inactivation of *Arf* tumor suppression in donor leukemia-initiating cells enables only 20 such polyclonal cells to induce lethal ALL in unconditioned syngeneic recipients within 30 days[12]. The use of this model allowed us to study the impact of host genetics on drug treatment responses without any potentially confounding variables accompanying irradiation or other conditioning of recipient animals.

In vivo treatment of leukemia

Treatment was started one day after injection of leukemia cells. To mimic clinically relevant treatment regimens[13], methotrexate was injected intraperitoneally (IP) at 1 mg/kg, once a week, as previously reported[5]. Mercaptopurine was administered in the drinking water. Assuming the mice drank 5 mL/day[14], the mice received 2 – 2.5 mg/kg/day of mercaptopurine at the 10 mg/L dose, 10–12.5 mg/kg/day at the 50 mg/L dose, and 20 – 25

mg/kg/day at the 100 mg/L dose. Septra was included in the drinking water at 600 mg/L sulfamethoxazole and 120 mg/L trimethoprim (daily doses of 120 – 150 mg/kg and 24 – 30 mg/kg, respectively).

Mice were sacrificed when they became moribund. Morbidity associated with ALL progression nearly always presented with hind limb paralysis, and often with a hunched back and/or domed head. Morbidity attributable to toxicity consisted of weight loss >20% of maximum body weight, anemia (pale paws and ears), and lethargy, with low ALL burden confirmed by bioluminescence and pathologic examination of formalin-fixed tissues. Mice sacrificed due to toxicity frequently were septic, with growth of *Klebsiella pneumoniae, Kocuria kristinae, Lactococcus garvieae, Enterococcus casseliflavus, Corynebacterium urealyticum*, Yeast spp., or *Corynebacterium urealyticum* from aerobic blood cultures. Gram staining and analysis (Vitek II Compact System, BioMerieux, Durham, NC) were used to identify the organisms from sterile blood cultures on blood agar or MacConkey agar plates.

At the time of sacrifice, blood was drawn by cardiac puncture while under isoflurane anesthesia into a tube with EDTA. Red blood cells were enumerated with a Coulter counter. The bones of the hind limbs were removed and the marrow was flushed with RPMI (Lonza BioWhittaker, Walkersville, MD). The marrow was macerated over a sterile cell strainer (40 um mesh) before counting cells with a hemacytometer. The spleen was excised and weighed. Tissues were fixed in 10% formalin, embedded in paraffin blocks, cut into 4 micron sections, and stained with hematoxylin and eosin.

Bioluminescent imaging

In vivo imaging was performed with the Xenogen IVIS-200 3 – 5 minutes post IP injection of 100 mg/kg D-luciferin (Caliper Life Sciences, Hopkinton, MA)[8]. The mice were anesthetized by isoflurane (2 – 3% in oxygen) prior to imaging. Images were acquired with small binning, beginning at one minute exposure, or less if images were saturated. Images were analyzed with Living Image 3.1 software (Caliper Life Sciences, Hopkinton, MA). Total flux measurements (photons/second) were quantified over the whole animal.

Statistical Analysis

Statistical analysis was done using Statistica 12 (Statsoft, Inc, Tulsa, OK). Survival curves were compared using the log-rank test. Other variables were compared using the Kruskal-Wallis test, Cochran-Armitage Trend Test or ANOVA. P values less than 0.05 were considered statistically significant.

Results

Efficacy of low dose mercaptopurine

Erythrocyte Tpmt activity differences among the *Tpmt* +/+, +/−, or −/− 129×1/SvJ mice were comparable to those recorded in patient erythrocytes[5]. After injection of 2000 BCR-ABL+ *Arf*-null *Tpmt* +/+ preB leukemia cells into male and female unconditioned, syngeneic mice, ALL was reproducibly lethal, with median survival (± standard error) similar among *Tpmt* genotypes in untreated mice: 16 (± 0.4) days for *Tpmt* +/+, 17 (± 0.7)

days for *Tpmt* +/−, and 18 (± 0.4) days for *Tpmt* −/− (Figure 1A). However, for the mice treated with low-dose mercaptopurine, the difference in 30-day leukemia-free survival was profound: 5% for *Tpmt* +/+ mice, 47% for *Tpmt* +/− mice, and 85% for *Tpmt* −/− mice ($p=5\times10^{-8}$, Cochran-Armitage Trend Test, Figure 1A). The cumulative leukemia-free survival of the *Tpmt* +/− mice treated with mercaptopurine was lower than the *Tpmt* −/− mice (p=0.001, log-rank) and trended higher than the *Tpmt* +/+ mice (p=0.075) treated with mercaptopurine, indicating a gene dosage effect for *Tpmt* on antileukemic efficacy. As expected, the *Tpmt* −/− mice suffered more frequent drug-induced toxicity than the other cohorts.

Untreated leukemic mice had elevated white blood cell (WBC) counts at the time of sacrifice (median 66,000/uL). In mercaptopurine-treated mice, the WBC count at day 21 differed by *Tpmt* genotype, with *Tpmt* −/− mice having the lowest, *Tpmt* +/+ mice having the highest, and *Tpmt* +/− mice having an intermediate phenotype (Figure 1B, p<0.000001). The mercaptopurine-treated *Tpmt* −/− mice sacrificed due to leukemia had a smaller spleen size than all other groups (p<0.000001, ANOVA), consistent with a superior antileukemic effect in the *Tpmt* −/− mice.

Disease in mice recapitulated characteristics of human ALL, with infiltration of leukemia cells in the blood, lungs, liver, spleen, lymph nodes, bone marrow, meninges and spinal column at the time of sacrifice (Figure 2). Mice with hind limb paralysis often displayed leukemic cells infiltrating subdurally from the vertebral marrow, which compressed the nerves surrounding the spinal cord.

In vivo bioluminescence was monitored once or twice weekly for assessment of leukemic burden. Whole-animal signal intensity was above 10^8 photons/second for all mice sacrificed due to leukemia. *Tpmt* +/− mice tended to have lower bioluminescence than *Tpmt* +/+ mice at day 21, while *Tpmt* −/− mice had the lowest (Figure 3A), indicating that the low dose of mercaptopurine had a greater salutary effect on the *Tpmt* +/− mice than the +/+ mice (Figure 3B, p=0.00001, ANOVA). Thus, haploinsufficiency for *Tpmt* results in an antileukemic response intermediate between the −/− and +/+ mice, although survival of the *Tpmt* +/− mice was more similar to the *Tpmt* +/+ mice than to the *Tpmt* −/− mice (Figure 1A).

Efficacy of higher doses of mercaptopurine

In order to confirm that greater efficacy could be achieved with higher doses of mercaptopurine in *Tpmt* +/+ mice, we increased the doses to 50 or 100 mg/L in groups of female mice. Antileukemic efficacy improved with higher doses of mercaptopurine, (Figure 4A, 100 mg/L vs 10 mg/L, p=0.001). The bioluminescence signal at day 15 after injection of leukemia (14 days of treatment) showed a dose-dependent effect: the leukemia burden was lowest in the mice receiving the highest dose of mercaptopurine (Figure 4B, p= 0.001, ANOVA). The cumulative leukemia-free survival of *Tpmt* +/+ mice treated with 100 mg/L mercaptopurine was equivalent to the *Tpmt* −/− mice treated with 10 mg/L (p=0.6, log-rank). The cumulative incidence of toxicity between these two groups was equivalent also (p=0.7, log-rank).

Discussion

The relationship between thiopurine-induced acute toxicity and *TPMT* genotype is well-established[3, 15–21]; however the relationship between *TPMT* genotype and antileukemic efficacy is less clear. Clinically, patients with heterozygous TPMT status (one wild-type and one inactive allele) constitute approximately 10% of most populations and have tolerance to thiopurines that is intermediate between those of homozygous deficient and homozygous wild-type patients, and intermediate starting doses of drug are recommended to minimize acute toxicity[4]. The impact of the host *TPMT* polymorphism on the desired antileukemic effects of thiopurines has been more difficult to assess clinically. Because TPMT homozygous deficient patients are so rare, the data on long-term cure rates in such patients are scarce, but it is clear that thiopurine doses must be reduced in such patients to avoid life-threatening toxicity, and anecdotally, such patients do not experience ALL relapse[16, 22]. Although some clinical studies indicate that patients who are heterozygous with respect to TPMT have lower ALL relapse rates when started at the usual high doses of mercaptopurine[18, 23, 24], other studies show little impact of TPMT status[17, 25–27], probably either because other elements of the ALL therapy overwhelmed the importance of thiopurines to cure, or because over the years, it has become more difficult to find clinical trials in which thiopurine doses were not decreased at least partly in response to data on TPMT status. In the current study, we were able to directly test the hypothesis that host *Tpmt* genotype influences the antileukemic efficacy of clinically relevant doses of mercaptopurine, even in the haploinsufficient state. We used a well-established murine model of BCR-ABL+ ALL[8, 28, 29] and treated with clinically relevant doses of oral mercaptopurine. This approach allowed us to segregate the impact of *Tpmt* status of the host versus the leukemia, because the leukemic cells were wild-type with respect to *Tpmt*. Because the *TPMT* status of somatic and germline cells are the same within a patient[30], such studies are only possible in genetically-tractable engineered murine models.

In clinical practice, the usual starting dosage of mercaptopurine has historically been driven by the fact that 90% of the population carries two wild-type alleles for *TPMT*, and thus a relatively high dose, often at 75 mg/m^2/day is administered[3, 17, 23, 31]. Such a dose, however, is completely intolerable clinically in the 0.25% of the population who inherit two inactive *TPMT* alleles, a condition which produces human “knock-outs” for *TPMT*, and which results in life-threatening myelosuppression [3]. Likewise, usual doses of mercaptopurine that are well tolerated by *Tpmt* +/+ mice result in early toxic deaths in *Tpmt* −/− mice[5]. Thus, our primary comparisons to test for the effect of *Tpmt* gene dosage on effectiveness was to use a dose low enough that all mice could tolerate, with 2 – 2.5 mg/kg/day for mice translating to approximately 7.2 – 9 mg/m^2 for a child[32], consistent with the low doses tolerated by homozygous deficient patients[3, 15, 19, 22, 33, 34]. However, we acknowledge that our dosing in the drinking water provides a more continuous source of mercaptopurine than the once per day dose normally used clinically, with some evidence that twice daily doses result in lower methylated metabolites than once daily dosing (albeit with no difference in TGN levels or outcome)[31, 35]. *Tpmt* +/− mice survived longer than *Tpmt* +/+ mice and had an intermediate level of leukemia at day 21, indicating that haploinsufficiency for *Tpmt* confers greater antileukemic effect in response to

a low fixed dose of mercaptopurine compared to that in *Tpmt* +/+ mice. The dose that was low enough for the *Tpmt* −/− mice to tolerate resulted in minimal anti-leukemic efficacy in the *Tpmt* +/+ mice. This is consistent with clinical data showing that low TPMT activity was associated with a lower risk of relapse[23], as well as with data showing that lowering doses in *TPMT* heterozygotes does not compromise cure rates[33, 36], and reflects their reduced minimal residual disease burden when fixed thiopurine doses are given[37]. The higher toxicity in the *Tpmt* −/− mice (at the same doses associated with greater antileukemic effect) illustrates the same difficulty faced clinically: doses must likely be pushed to the point of some myelosuppression in order to achieve optimal antileukemic effects. When the *Tpmt* +/+ mice were treated with higher doses, they also experienced toxicity but displayed an improved antileukemic effect. These findings are consistent with recent clinical recommendations[4] to use three different starting doses for mercaptopurine based on *TPMT* status (wild-type > heterozygous > homozygous deficient), and then titrate to the desired degree of myelosuppression. Using this approach, cure rates in trials for treating childhood ALL were not compromised[33, 36].

In summary, we've demonstrated that in an engineered and genetically-defined murine leukemia model that mimics the human genetic germline polymorphism in *TPMT*, host *Tpmt* genotype and phenotype influenced the antileukemic efficacy of mercaptopurine, and that haploinsufficiency (heterozygous state equivalent for humans) confers a risk intermediate between homozygous wild-type and homozygous deficient status. These data are consistent with the increasingly accepted clinical practice of reducing the dose of thiopurines in TPMT +/− and −/− patients to avoid host toxicity while maintaining antileukemic efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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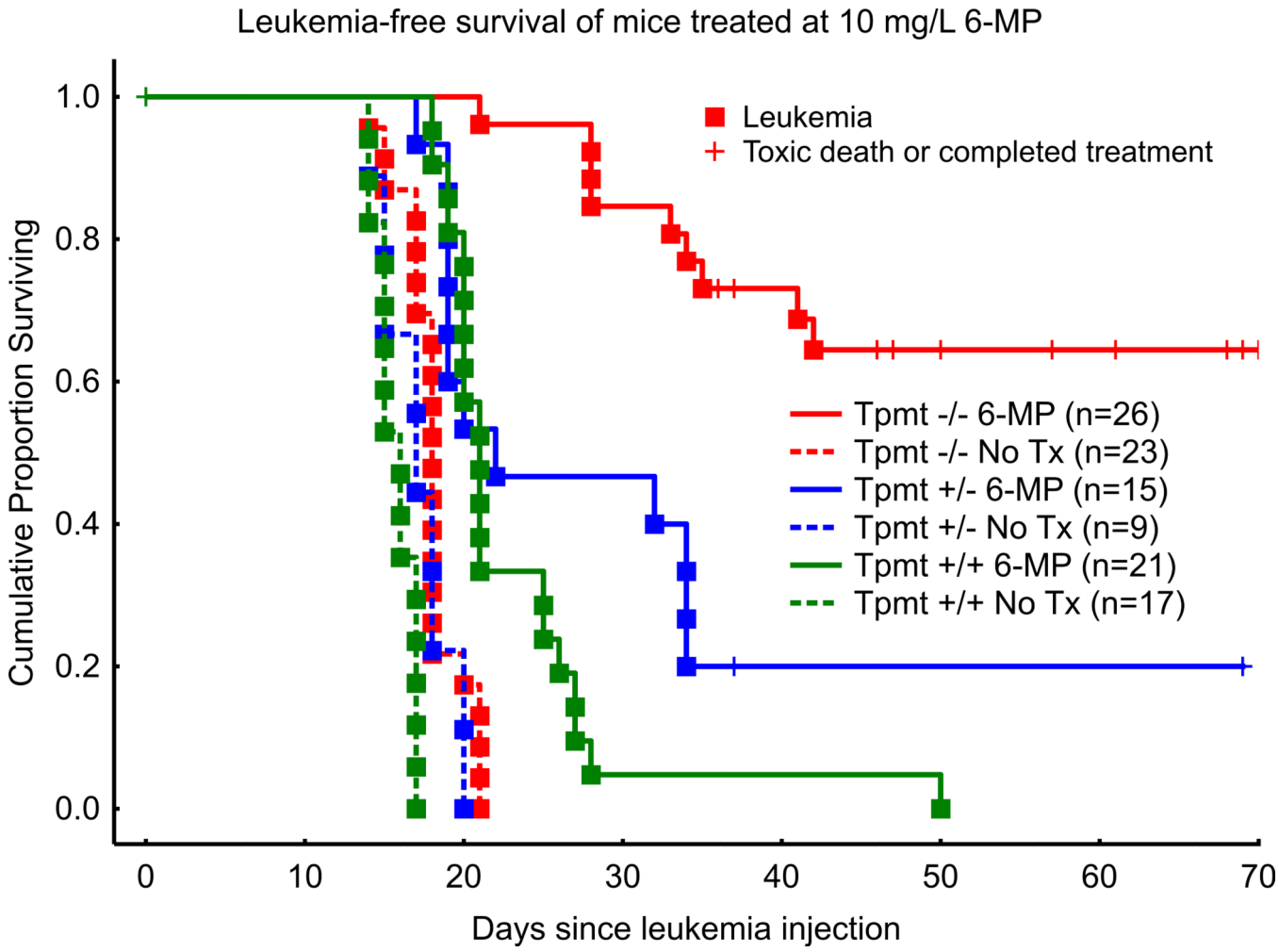


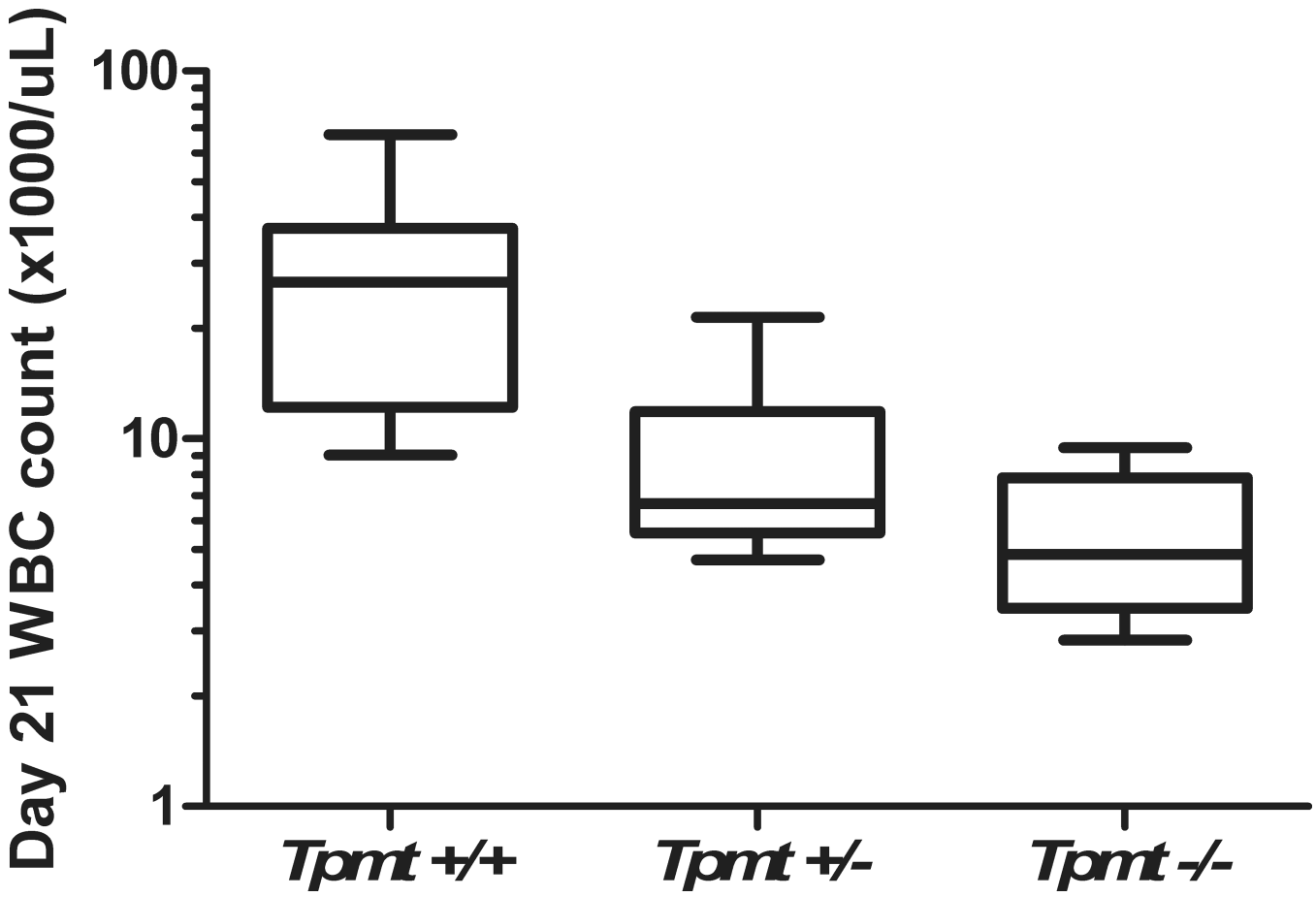


Figure 1.

Survival and leukemic burden by *Tpmt* genotype. Male and female unconditioned mice were injected IV with 2,000 *Arf*-null BCR-ABL+ leukemia cells and treated with 10 mg/L mercaptopurine in the drinking water and 1 mg/kg/week methotrexate delivered IP beginning day 1 after leukemia injection. **A**, Survival of all mice injected with leukemia, both treated (solid lines) and untreated (dotted lines), colored by genotype. P-values are based on the log-rank test comparing the two groups indicated: *Tpmt*−/− 6-MP vs *Tpmt*+/− 6-MP, p=0.001; *Tpmt*−/− 6-MP vs *Tpmt*+/+ 6-MP, $p<10^{-6}$; *Tpmt*+/− 6-MP vs *Tpmt*+/+ 6-MP, p=0.075. **B**, White blood cell counts at day 21 after injection in mercaptopurine-treated mice injected with leukemia, grouped by *Tpmt* genotype, show a gene dosage effect on antileukemic efficacy (p<0.000001, Kruskal-Wallis). Boxes represent 25^{th}– 75^{th} percentile, horizontal line represents the mean, whiskers indicate minimum to maximum values.

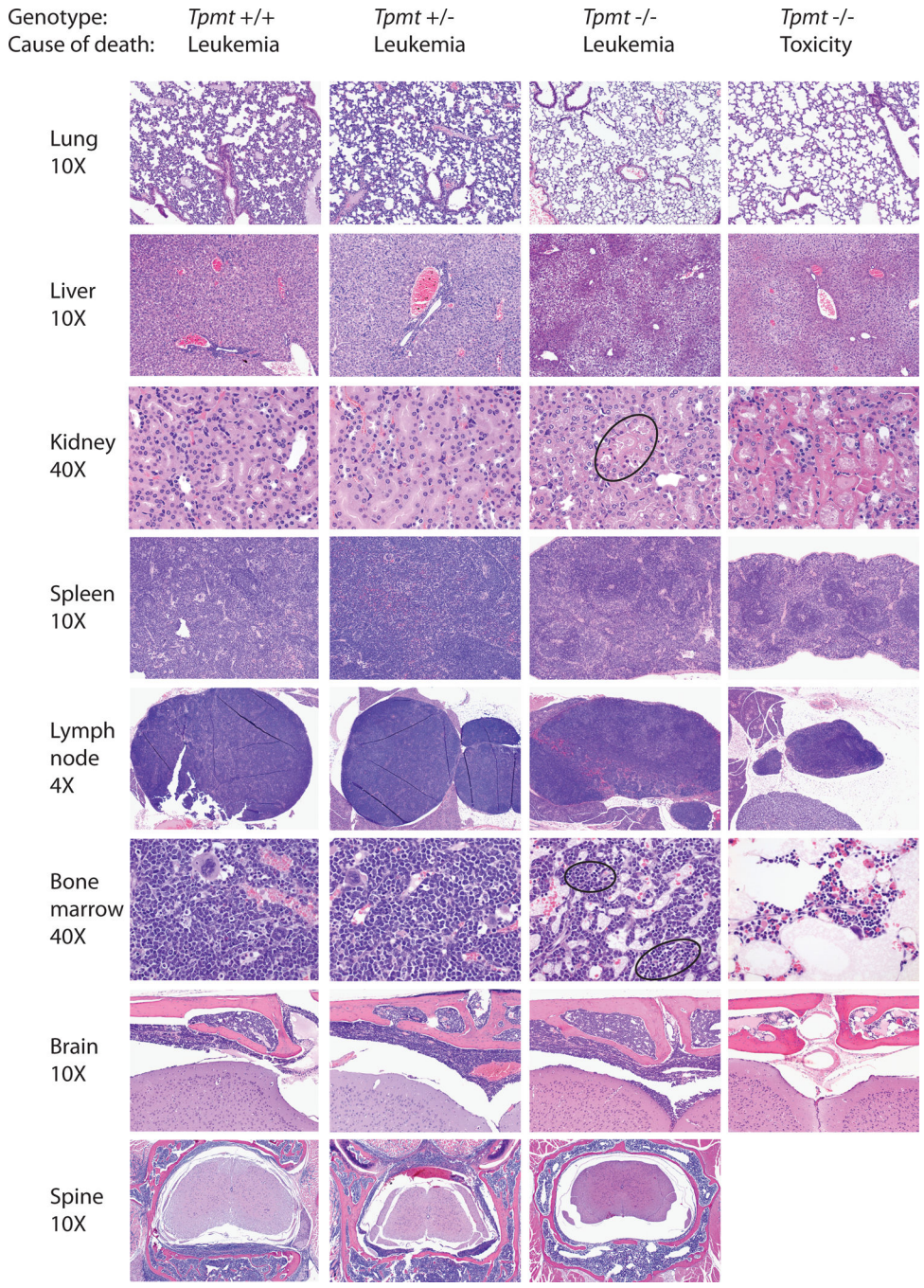
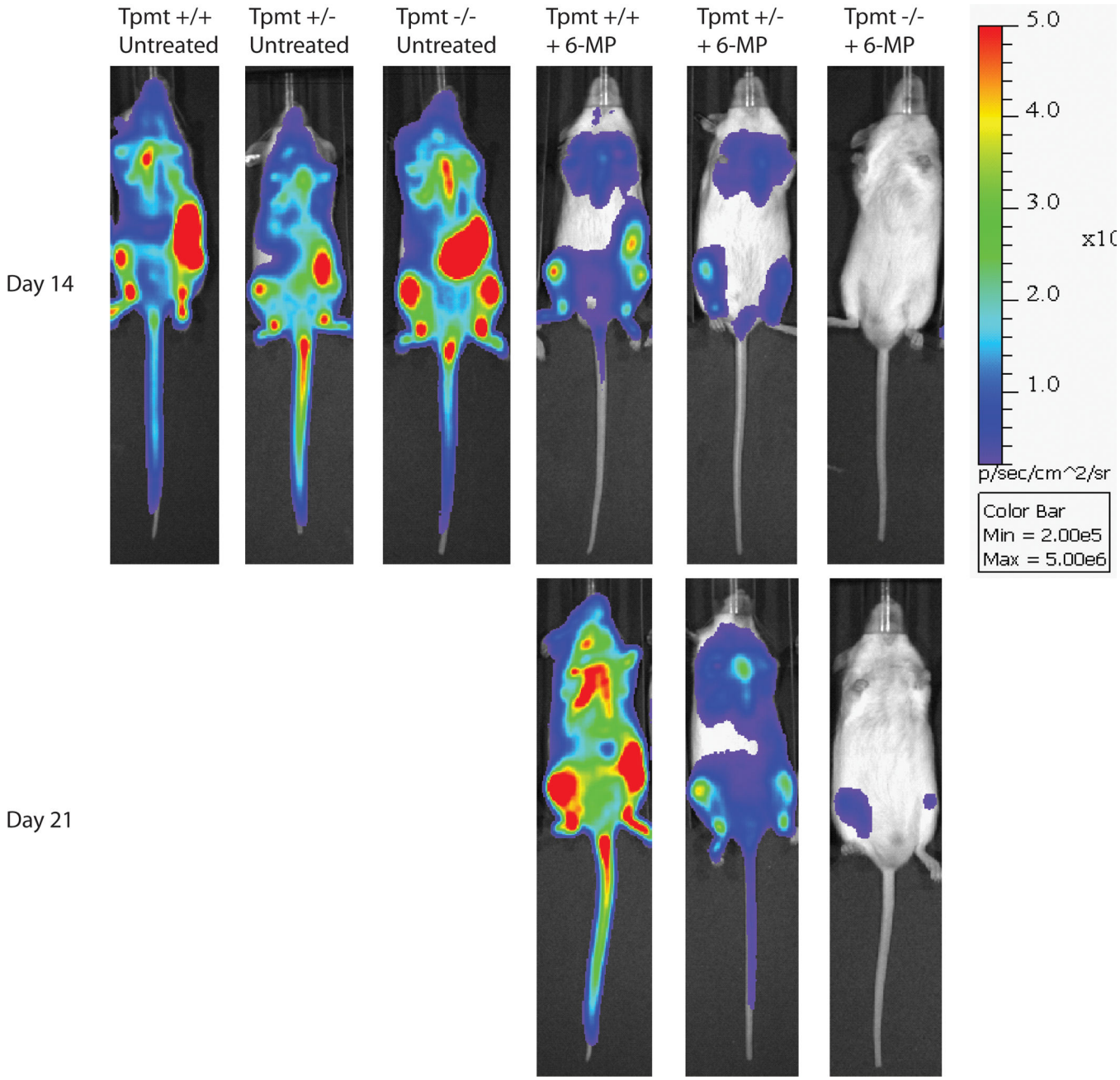


Figure 2.

Hematoxylin and eosin stained tissue sections obtained at the time of sacrifice from a representative mouse from all three genotypes for whom cause of death was leukemia (first 3 columns) or toxicity (right column). Mice were injected with 2,000 *Arf*-null BCR-ABL+ leukemia cells, treated with 10 mg/L mercaptopurine in the drinking water and 1 mg/kg/week methotrexate delivered IP beginning day 1 after injection. **Lung, 10×**. *Tpmt* +/+ & +/−: alveolar septae thickened by leukemia cells; *Tpmt* −/− leukemia: essentially normal. *Tpmt* −/− toxicity: essentially normal. **Liver, 10×**. *Tpmt* +/+ & +/−: leukemia cells surround blood

vessels and are within sinusoids. *Tpmt* −/− leukemia: moderate hepatocellular degeneration. *Tpmt* −/− toxicity: mild degeneration. **Kidney, 10×**. *Tpmt* +/+ & +/−: essentially normal. *Tpmt* −/− leukemia: few very small areas of necrotic tubules (circled area); this is more extensive in *Tpmt* −/− toxicity (entire image). **Spleen, 10×**. *Tpmt* +/+ & +/−: Almost completely replaced by leukemia cells. *Tpmt* −/− leukemia: less severe replacement by leukemia cells. *Tpmt* −/− toxicity: almost normal, slightly small spleen. **Lymph nodes, 4×**. *Tpmt* +/+, +/−, & −/−: Almost completely replaced by leukemia cells. *Tpmt* −/− toxicity: almost normal, slightly small lymph node. **Bone marrow, 40×**. *Tpmt* +/+ & +/−: almost completely replaced by leukemia cells, few megakaryocytes remaining. *Tpmt* −/− leukemia: leukemia cells not as densely filling the marrow space, and a few foci of erythroid precursors remain (circled). *Tpmt* −/− toxicity: severe marrow hypocellularity/toxicity. **Brain, 10×**. *Tpmt* +/+, +/−, & −/− leukemia: leukemia cells fill cranial marrow and extend into meninges. *Tpmt* −/− toxicity: normal. **Spine, 10×**. *Tpmt* +/+, +/−, & −/− leukemia: leukemia cells in vertebral marrow. Epidural infiltration more severe in *Tpmt* +/+ & +/−. Image not available for *Tpmt* −/− toxicity.



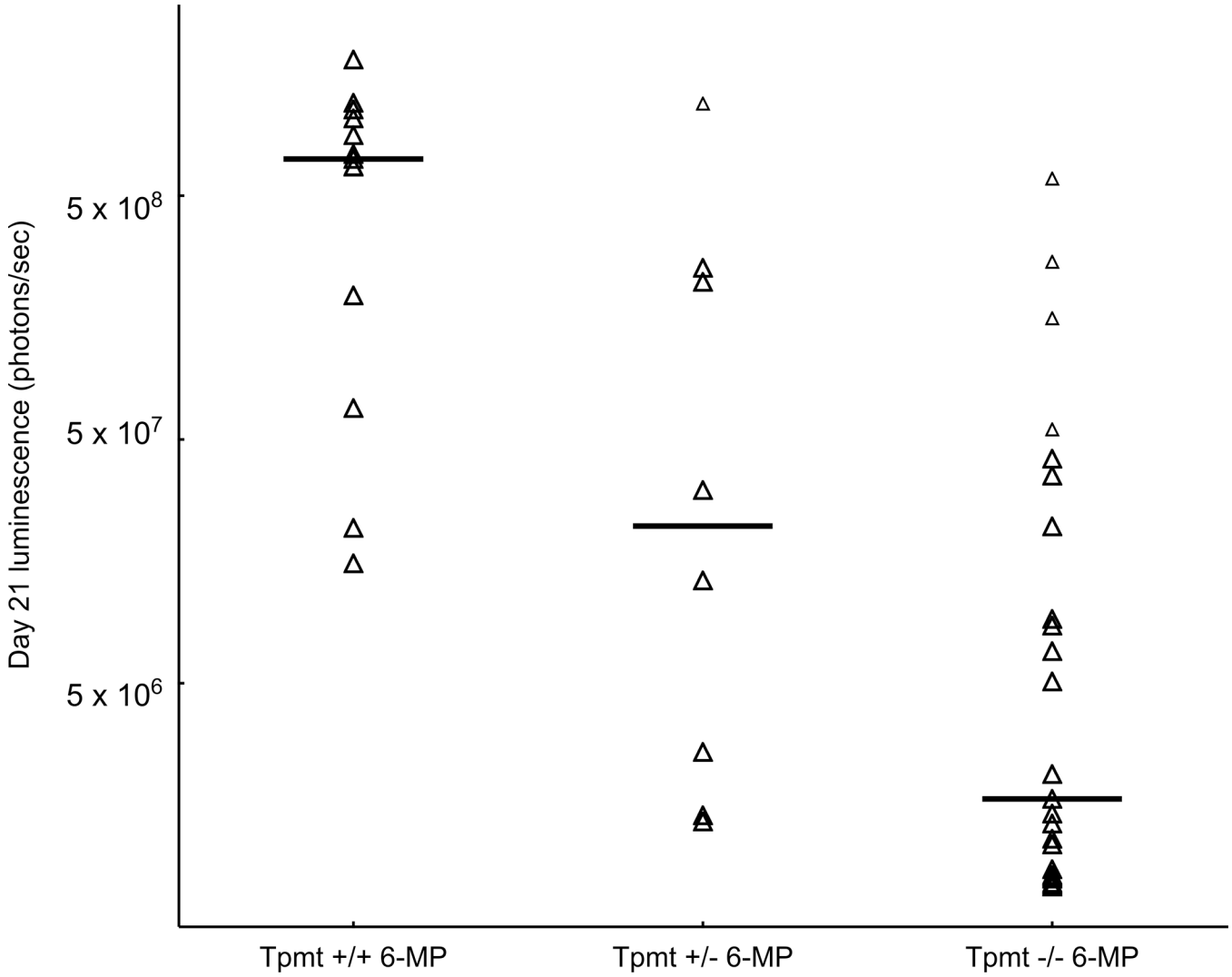
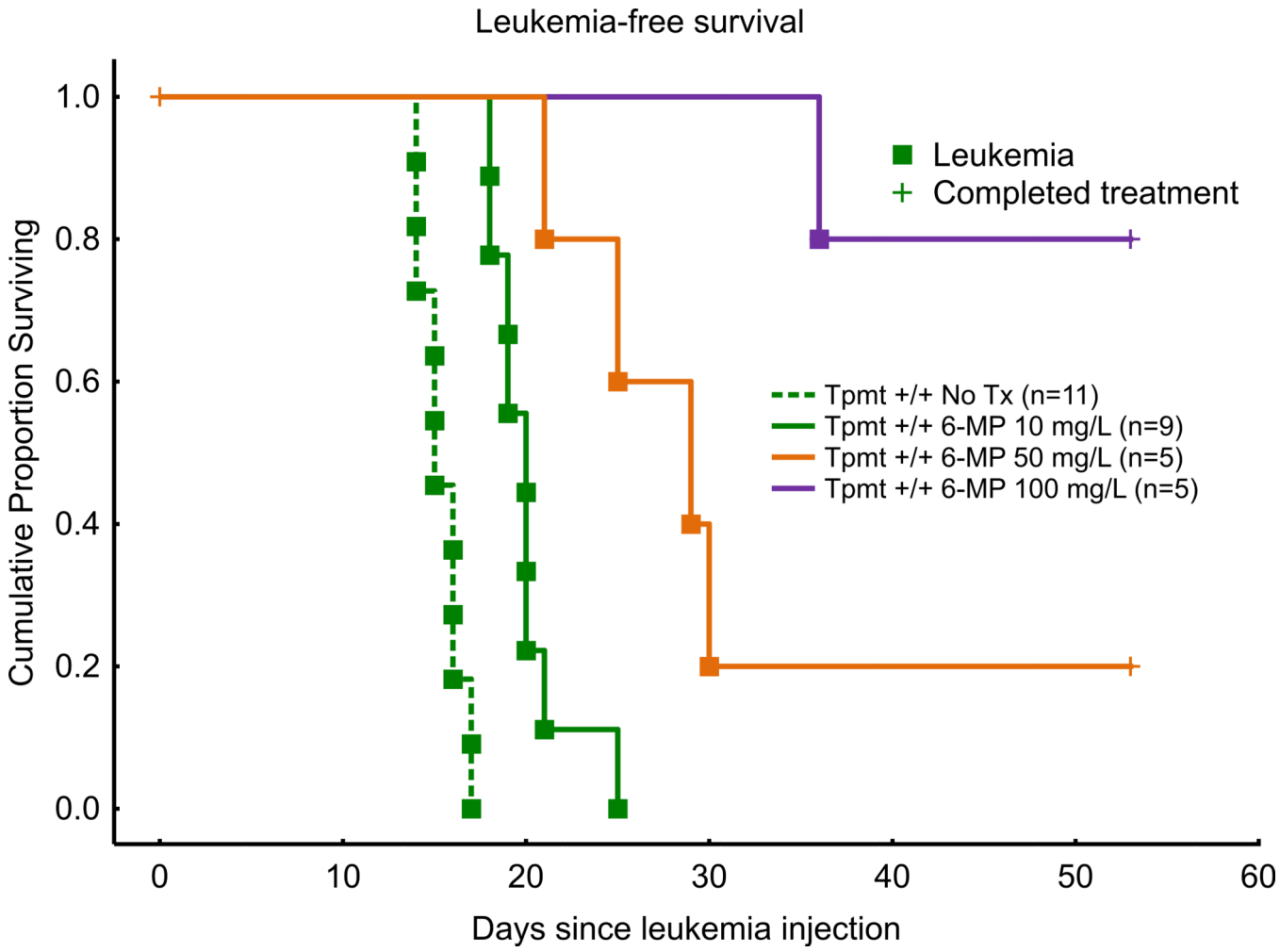


Figure 3.

In vivo leukemic burden at days 14 and 21. Mice were injected with 2,000 *Arf*-null BCR-ABL+ leukemia cells, treated with 10 mg/L mercaptopurine in the drinking water and 1 mg/kg/week methotrexate delivered IP beginning day 1 after injection. **A**, Bioluminescence for a representative mouse in each group is shown for days 14 and 21 after injection. **B**, Bioluminescence for mercaptopurine-treated mice at day 21; untreated mice were sacrificed due to leukemia progression between day 14 and day 21. The horizontal line represents the median. ANOVA p=0.00001. The maximum signal intensity for each image was set to 5×10^6 p/sec/cm^2/sr, and the minimum signal intensity was set to 2×10^5 p/sec/cm^2/sr. The binning was set to 4 and the smoothing set to 3×3.



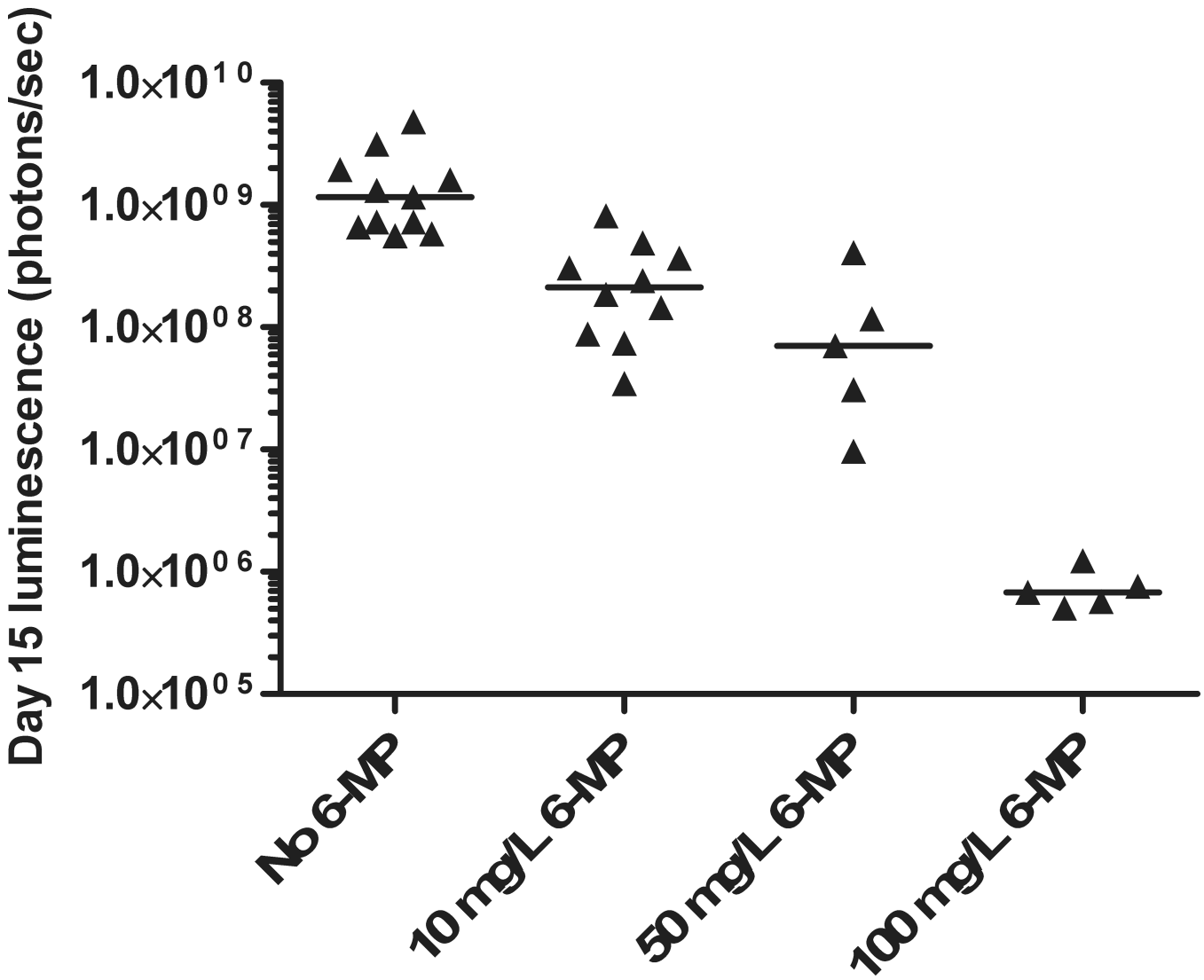


Figure 4.

Leukemia-free survival is improved in *Tpmt* +/+ treated with 100 mg/L mercaptopurine. **A**, Leukemia-free survival of female *Tpmt* +/+ mice injected with 2,000 *Arf*-null BCR-ABL+ leukemia cells and treated with no 6-MP or 6-MP starting at day 1 at 10 mg/L, 50 mg/L, or 100 mg/L. **B**, In vivo luminescence of *Tpmt* +/+ female mice injected with 2,000 *Arf*-null BCR-ABL+ leukemia cells and treated with no 6-MP or 6-MP at 10 mg/L, 50 mg/L, or 100 mg/L. Horizontal lines represent the median, raw data are indicated by triangles.