

**EFFECT OF IMATINIB ON THE REPRODUCTIVE FUNCTION
IN MALE SWISS ALBINO MICE**

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Summary

Tyrosine kinase inhibitors may cause transient or permanent testicular dysfunction when used in treatment of cancer. Like other anticancer agents tyrosine kinase inhibitors are genotoxins and induce irreversible effect on genetic makeup and may have carcinogenic potential. This is alarmingly dangerous in youth and children, since these effects last longer, affecting fertility or forming basis for carcinogenesis. There is paucity of reports on planned study of imatinib on testicular function. Hence the study was planned to assess the effects of imatinib on sperm morphology in male swiss albino mice. *Methods:* Male swiss albino mice were treated with imatinib and sacrificed at the end of 1st, 2nd, 4th, 5th, 7th and 10th week after the last exposure to imatinib. Testes were removed; cauda epididymis was separated and processed for sperm morphology assay. *Results:* There was significant but reversible decrease in sperm motility and count. There was a significant increase in abnormal sperms with all the doses of imatinib. *Conclusion:* Imatinib does affect sperm morphology significantly, but this effect is reversible once the drug is withdrawn.

Key words: imatinib, sperm motility, sperm count.

Introduction

The improvements achieved in diagnosis and therapy in oncology has led to a significantly increasing survival of young patients affected by malignancies. Today, nearly 85% of tumors in children and young men can be successfully attacked (1, 2). However, the long-term treatments can cause infertility. Semen analysis is the initial and most essential step of the infertility evaluation, which also includes a physical exam, hormonal evaluation, sperm function testing, and genetic analysis. It is also considered a cornerstone of the laboratory evaluation of the infertile male and helps define the severity of male factor infertility (MFI).

Imatinib mesylate, a first synthetic tyrosine kinase inhibitor, inhibits proliferation and induces apoptosis in Bcr-Abl positive cell lines as well as fresh leukemic cells from Philadelphia chromosome positive chronic myeloid leukemia. It is indicated for the treatment of patients with chronic myeloid leukemia. Imatinib (formerly known as STI571) has demonstrated high levels of efficacy in gastrointestinal stromal tumors (3). There is a report of gastrointestinal stromal tumor (GIST) patient with male gynecomastia and testicular hydrocele after treatment with imatinib mesylate (4). Also reports are available for gynecomastia in men with chronic myeloid leukaemia after imatinib (5). But the report on effect of imatinib on reproductive function is scanty. There is paucity of reports on planned study of imatinib on testicular function. Hence a study was planned to assess the effects of imatinib on reproductive functions in male swiss albino mice.

Materials & Methods:

Male Swiss albino mice (9-12 week old) were used. Animals were bred in the central animal house of Manipal University, Manipal. Breeding and maintenance of animals were done according to the guidelines of Committee for the purpose of control and supervision of experiments on animals (CPCSEA) and Animal Welfare Division, Government of India for the use of laboratory animals. This work was carried out after obtaining approval from the institutional animal ethical committee. All the animals were housed in polypropylene cage using paddy husk for bedding at $28\pm 1^{\circ}\text{C}$ temperature and $50\pm 5\%$ humidity. Five animals were housed in each cage to prevent overcrowding. Animals were fed on laboratory feed (Gold Mohur; Lipton India Ltd) and water *ad libitum*.

The animals were segregated into 24 groups containing 6 animals in each group. Eighteen groups were injected with Imatinib at the dose levels of 50, 75 and 100 mg/kg body weight (6 groups for each dose) intraperitoneally for a continuous period of five days with an interval of 24 hours between two injections. Remaining six groups served as controls, which received distilled water. After the last dose, the animals were sacrificed on 1, 2, 4, 5, 7 and 10 weeks sample times by overdose of anesthesia (Pentobarbital sodium, 40mg/kg, Sigma Chemicals Co). These sample time (1-5 weeks) establishes the treatment of spermatozoa in the epididymis and testis, spermatids, primary spermatocytes, secondary spermatocytes, spermatogonia and 7,10 week sample time represents treatment as stem cells respectively (6,7,8).

Sperm morphology assay

The mice were sacrificed at different week samples and laporatomy was done. Testes were removed; cauda epididymis was separated (Testes were processed for histopathological sections and tissue homogenization). Sperm suspensions were prepared by mincing cauda in 2ml of phosphate buffered physiological saline (PBS, pH=7.2). Suspension was pipetted and filtered through 80 μm nylon mesh to remove tissue fragments. A fraction of suspension was then mixed with (10:1) with eosin Y and 30 minutes later about one drop of stained suspension was placed on the clean slide. It's dried, cleaned and mounted in DPX. Slides were looked for sperm shape abnormality. Slides were coded for blind analysis.

From each suspension 1000 sperms were examined at 400X with blue-green filter. Abnormal sperms are classified as, I. Head abnormality- that included: hook less, banana shaped, double headed and amorphous. II. Tail abnormality- which includes the coiled and double tailed sperms.

Epididymal sperm count and sperm motility

Mice were sacrificed by overdose of anesthesia; Testes were removed, cauda epididymis was separated, (the suspension obtained from the same animal sample has been used to determine sperm morphology assay) and sperm numbers per epididymis were determined by haemocytometer.

An Aliquot (0.05ml) from the sperm suspension (1ml) was diluted with 1:40 phosphate buffered saline (PBS, pH 7.2) and mixed thoroughly. After discharging few drops, a sample of the diluted sperm suspension was introduced into a counting chamber. The total sperm count in 8 squares of 1mm² each was determined and multiplied by 5×10⁴ to calculate the number of sperms per epididymis. Sperm motility was also counted in same eight squares and percentage of motile sperm were recorded

Statistical analysis:

For each group six animals were used and mean ± SD (standard deviation) was calculated. Results obtained from the present study were correlated and analyzed by one way Analysis of Variance (ANOVA) followed by Bonferroni's post hoc test. Values of *P*<0.05 were considered statistically significant.

Results**Effect of imatinib on sperm count**

Imatinib had a significant affect on the sperm count at each of the treated doses. On the first week sampling time, each dose of the drug (50mg/kg, 75mg/kg & 100mg/kg) considerably decreased sperm count in mice when compared to the control groups. Similar drop in sperm count was also seen through the 2nd, 4th and 5th week sampling time. For the mice treated with 50mg/kg and 75mg/kg, sperm count was least during the 2nd week sampling time and for 100mg/kg, sperm count was least during 5th week. However the sperm count returned closer to control levels in two dose groups (50mg/kg and 75mg/kg) by the 10th week (Table 1 and Figure 1). The recovery was almost the same in both lower doses (50mg/kg, 75mg/kg) which was initiated in 7th week and reached close to the control group by 10th week. But the higher dose group did not show complete recovery.

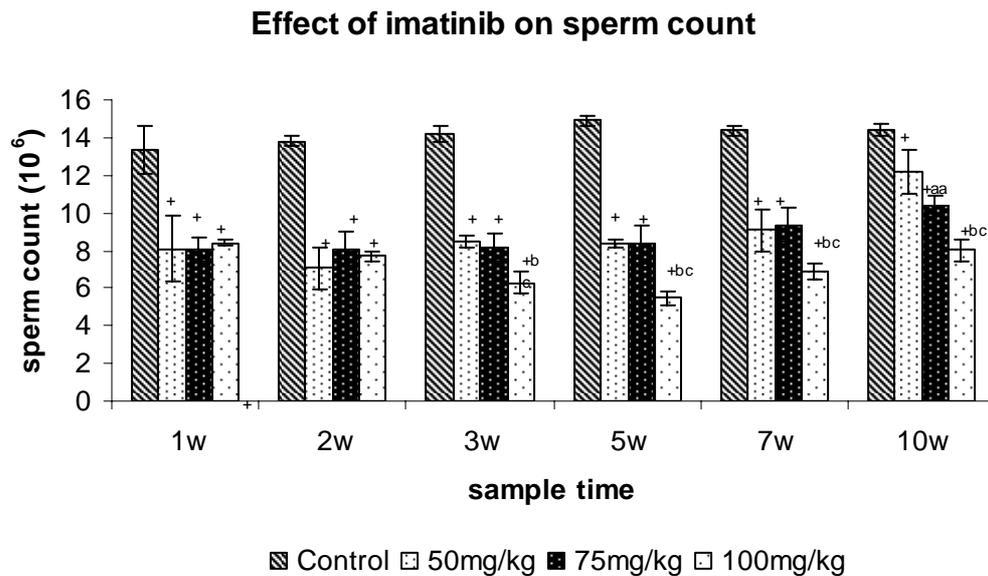


Fig 1. Effect of imatinib on sperm count (10^6). Each dose from particular time represents mean \pm SD from 6 animals. p values are control vs treated, +<0.001; 50 mg/kg vs 75 mg/kg, aa<0.01; 50 mg / kg vs 100 mg/kg, b<0.001; 75 mg / kg vs 100 mg/kg, c<0.001. w=weeks.

Dose	Sampling Time					
	1w	2w	3w	5w	7w	10w
Control	13.4 \pm 1.27	13.8 \pm 0.29	14.2 \pm 0.38	14.9 \pm 0.24	14.4 \pm 0.26	14.4 \pm 0.34
50mg/kg	8.1 \pm 1.72 ⁺	7.05 \pm 1.13 ⁺	8.5 \pm 0.31 ⁺	8.4 \pm 0.21 ⁺	9.09 \pm 1.12 ⁺	12.2 \pm 1.13 ⁺
75mg/kg	8.1 \pm 0.64 ⁺	8.1 \pm 0.86 ⁺	8.19 \pm 0.72 ⁺	8.32 \pm 0.97 ⁺	9.34 \pm 0.91 ⁺	10.4 \pm 0.49 ^{aa}
100mg/kg	8.4 \pm 0.16 ⁺	7.7 \pm 0.26 ⁺	6.3 \pm 0.60 ^{+bc}	5.5 \pm 0.38 ^{+bc}	6.9 \pm 0.46 ^{+bc}	8.01 \pm 0.58 ^{+bc}

Table 1. Effect of imatinib on sperm count (10^6). Each dose from particular time represents mean \pm SD from 6 animals. p values are control vs treated, +<0.001; 50 mg/kg vs 75 mg/kg, aa<0.01; 50 mg / kg vs 100 mg/kg, b<0.001; 75 mg / kg vs 100 mg/kg, c<0.001. w=weeks.

Effect of imatinib on sperm motility

Sperm motility was significantly decreased during the 1st, 2nd, 4th, 5th and 7th week sampling time with all treated doses of the drug (50mg/kg, 75mg/kg and 100mg/kg). For the mice treated with 50mg/kg sperm motility was least during the 3rd week sampling time, for 75mg/kg and 100mg/kg, sperm motility was least during the 5th week sampling time (Table 2 and Figure 2). By the 10th week there was complete recovery and the sperm motility values reached the control values except the higher dose group (100mg/kg).

Effect of imatinib on sperm motility

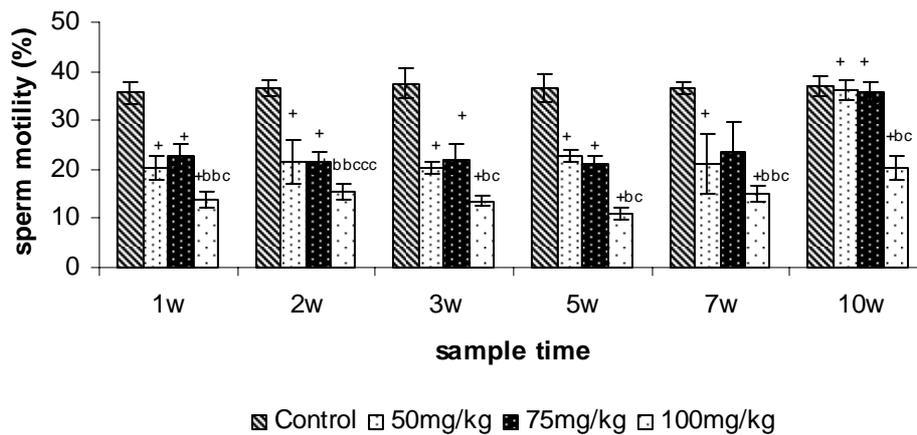


Fig 2.Effect of imatinib on sperm motility(%). Each dose from particular time represents mean \pm SD from 6 animals. p values are control vs treated, +<0.001;; 50 mg / kg vs 100 mg/kg, bbb<0.05, bb<0.01, b<0.001; 75 mg / kg vs 100 mg/kg, ccc<0.05, c<0.001. w=weeks.

Dose	Sampling Time					
	1w	2w	3w	5w	7w	10w
Control	35.6 \pm 2.35	36.5 \pm 1.70	37.6 \pm 2.86	36.5 \pm 2.87	36.6 \pm 1.37	37.0 \pm 2.00
50mg/kg	20.3 \pm 2.28 ⁺	21.4 \pm 4.52 ⁺	20.3 \pm 1.37 ⁺	22.8 \pm 1.37 ⁺	21.3 \pm 6.09 ⁺	38.6 \pm 2.01 ⁺
75mg/kg	22.8 \pm 2.28 ⁺	21.5 \pm 2.21 ⁺	22.1 \pm 2.97 ⁺	21 \pm 1.71 ⁺	23.6 \pm 6.00 ⁺	35.6 \pm 2.02 ⁺
100mg/kg	13.8 \pm 1.57 ^{+bbc}	15.5 \pm 1.70 ^{+bbbccc}	13.6 \pm 1.15 ^{+bc}	11 \pm 1.15 ^{+bc}	15.1 \pm 3.97 ⁺	20.3 \pm 2.28 ^{+bc}

Table 2. Effect of imatinib on sperm motility(%). Each dose from particular time represents mean \pm SD from 6 animals. p values are control vs treated, +<0.001;; 50 mg / kg vs 100 mg/kg, bbb<0.05, bb<0.01, b<0.001; 75 mg / kg vs 100 mg/kg, ccc<0.05, c<0.001. w=weeks.

Effect of imatinib on sperm morphology

The incidence of abnormal sperms showed significant increase during the 1st week sampling time in mice treated with high dose (100mg/kg) when compared to the control group. However the percentage of abnormalities increased in a time dependent manner during the 2nd, 4th and 5th week sampling time in mice treated with all the doses. The maximum sperm abnormality was observed during the 5th week sampling time in mice treated with all the doses (Table 3 and Figure 3). The higher dose group showed the maximum percentage of abnormal sperms during the most week sampling time except week 10 which showed a value closer to the control group. Although maximum sperm abnormalities were seen during the 5th week sampling time in mice treated with 50mg/kg and 75mg/kg, the percentage of abnormal sperms were still less compared to the mice treated with 100mg/kg. The recovery period was similar for all the treated groups of mice. The percentage of abnormal sperms reached closer to the control values in mice treated with all the doses of the drug during the 10th week sampling time.

Effect of Imatinib on sperm morphology

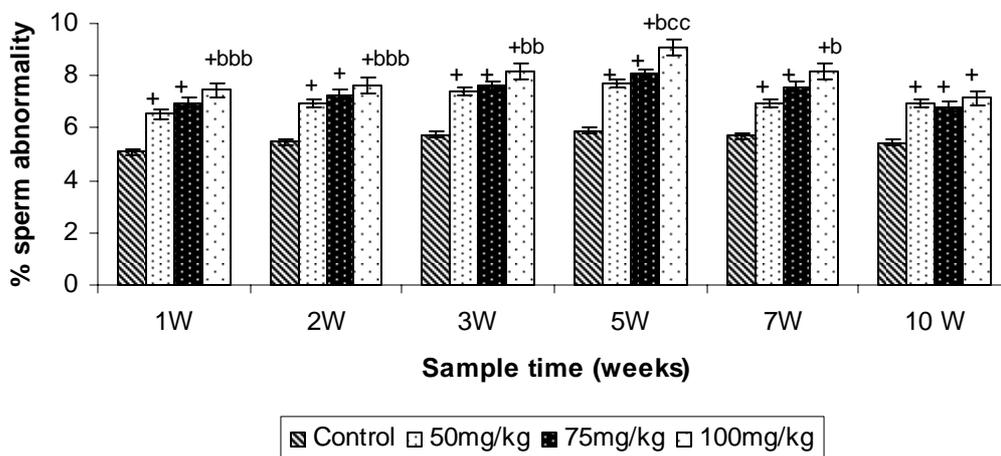


Fig 3.Effect of imatinib on sperm morphology(%). Each dose from particular time represents mean ±SD from 6 animals. p values are control vs treated,+<0.001; 50 mg / kg vs 100 mg/kg, bbb<0.05, bb<0.01, b<0.001; 75 mg / kg vs 100 mg/kg, cc<0.01, c<0.001. w=weeks.

Dose	Sampling Time					
	1w	2w	3w	5w	7w	10w
Control	5.08 ± 5.30	5.46 ± 2.85	5.73±2.13	5.91 ± 1.95	5.7 ± 2.38	5.45 ± 3.25
50mg/kg	6.53 ± 4.71 ⁺	6.94 ± 3.58 ⁺	7.41 ± 4.41 ⁺	7.7 ± 6.8 ⁺	6.93± 4.57 ⁺	6.93 ± 4.10 ⁺
75mg/kg	6.98 ± 3.57 ⁺	7.28 ± 3.90 ⁺	7.6 ± 2.64 ⁺	8.08 ± 2.6 ⁺	7.58 ± 4.91 ⁺	6.81 ± 3.57 ⁺
100mg/kg	7.45 ± 3.73 ^{bbb}	7.62 ± 2.97 ^{bbb}	8.18 ± 3.48 ^{bb}	9.1 ± 3.65 ^{bcc}	8.18 ± 3.48 ^b	7.15 ± 1.97 ⁺

Table 3.Effect of imatinib on sperm morphology(%). Each dose from particular time represents mean ±SD from 6 animals. p values are control vs treated,+<0.001;; 50 mg / kg vs 100 mg/kg, bbb<0.05, bb<0.01, b<0.001; 75 mg / kg vs 100 mg/kg, cc<0.01, cp<0.001. w=weeks.

Discussion

The decrease in sperm density in the epididymis is an indicator of reduced spermatogenesis owing to the toxicity of any agent (9). In fact, the male rat fertility is not compromised until the sperm count is reduced to less than 10% of the normal values, whereas a decrease to 30% is sufficient to result in considerably reduced fertility in human. Furthermore, the human male has a relatively low fertility and thus is at greater risk from reproductive toxicants than are males of laboratory animal species (10). In our study imatinib was given intraperitoneally for 5 days to estimate the sperm count. Our results show that imatinib was cytotoxic to the sperm since it decreased the sperm count significantly in a linear manner from 1st to 5th week sampling time, regardless of the dose. From our findings we can point out that the germ cells affected are approximately the spermatids, spermatocytes and spermatogonia. Complete recovery was observed by the 10th week in the lower dose groups (50mg/kg, 75mg/kg). Nurmio et al (11) reported that repeated dosage of imatinib inhibited migration process of centrally located germ cells. Imatinib blocked or delayed migration of gonocytes from the centre of the seminiferous cords to the basement membrane to form spermatogonial stem cell pool. Inhibition of migration was most probably due the blockage of c-kit receptor, as the observation is in a good agreement with the earlier finding that the presence of c-kit antiserum in Sertoli cell-gonocyte co-culture inhibits migration of gonocytes (12).

In our result there was a decrease in the sperm count which may be explained by the inhibition of migration of sperm due to the blockage of c-kit receptor. Even though mice treated with imatinib showed significant decrease in the sperm count at the end of 7th week, it was not mutagenic to the sperms at that time. This can be demonstrated by the reduced number of abnormal sperms observed by the end of that week. This point to the fact that the reduction in sperm counts could be due to the reduction in the intratesticular testosterone levels. It has recently been found that suppression of the blood levels of follicle-stimulating hormone (FSH) in the critical period before day 15 inhibits Sertoli cell multiplication (13). If Sertoli cell multiplication is altered transiently, it results in adulthood with parallel reductions in Sertoli cell number, testicular weight and sperm output. The number of sperms produced is affected but the process of spermatogenesis remains normal. Imatinib induces qualitative and quantitative deteriorations of spermograms in rats (14). The decrease in the sperm count in the present study may be due to the decreased levels of intratesticular testosterone at the 2nd to 7th week sampling time (result not shown). It is also possible that the Sertoli cells might have been affected and the other possibility might be due its effect on the epididymal function. The dose - and time - dependent decrease in the number of sperm following imatinib treatment noted in the present study is an indication of depressed spermatogenesis due to the inhibition of cell multiplication and interference by genetically controlled programme of sperm differentiation process. This study indicates that imatinib affects spermatogenesis by causing depression in sperm count, but this effect is reversible once the drug is withdrawn which indicates that the stem cells were not affected severely.

Sperm motility assessments are an integral part of some reproductive toxicity test guidelines (15, 16, and 17). Bieber et al. (18) reported that the sperm tail showed abnormalities of mitochondrial sheath, central and peripheral doublets as well as outer dense fibers. These changes in tail morphology would be responsible for abnormal sperm motility. Eddy, E. M et al (19) analyzed that the midpiece contains the tightly packed helical array of mitochondria, and the principal piece is defined by the presence of a fibrous sheath. So any damage affecting the midpiece of sperm tail can result in decreased sperm motility. Since endogenous cannabinoids like AEA reduce, in a dose-dependent manner, mitochondrial activity in human sperm (20).

Dawson et al. (21) found that high concentrations of aluminium in human spermatozoa and seminal plasma are correlated with decreased sperm motility and viability. The observed decrease in sperm motility could be attributed in part to the concomitant reduction in testosterone production (22). The increase in TBARS can bring negative effects on motility, midpiece abnormalities and sperm–oocyte fusion (23). In our result, sperm motility was significantly decreased during the 1st, 2nd, 4th, 5th and 7th week sampling time with all treated doses of the drug (50mg/kg, 75mg/kg and 100mg/kg). By the 10th week there was complete recovery and the sperm motility values reached the control values except the higher dose group (100mg/kg). It is clear from our result that the sperm motility was least during 5th week in high dose groups. It is also noted that the maximum sperm abnormality was observed during the 5th week sampling time in mice treated with all the doses. The sperm motility largely depends on the microtubular apparatus of the sperm tail. In the current study it was also observed that a considerable number of abnormal sperms were with a defect in their tail. Mitochondria are needed for the energy production of the cells and motility of the sperm requires normal mitochondrial function. (24). It could be possible that due to the tail abnormalities resulted by the imatinib toxicity, the mitochondrial function might have been affected which may lead to decreased motility.

Our present study shows that, imatinib is genotoxic to the germ cell. The percentage of sperm abnormalities increased in a time dependent manner during the 2nd, 4th and 5th week sampling time in mice treated with all the doses. The structure of mature sperm consists of a head and a long flagellum (25). The tail is divided into four distinct segments: the connecting piece adjacent to the head, the midpiece, and the principal and end pieces. Some authors believe that sperm abnormalities are the resultant end points after point mutations or other chromosome variations (26, 27). It is possible that these changes in the sperm structure might have been due to point mutation. The maximum sperm abnormality was observed during the 5th week sampling time in mice treated with all the doses. . It is essential that, to label any drug as mutagen, it should induce double the sperm abnormality compared to the control level (26). Imatinib could be judged as a germ cell mutagen, since it could induce more than double of abnormal sperms compared to control, a criterion required to be fulfilled for any agent to be categorized so, based on sperm morphology test. Their mutagenic effect was more pronounced during the 1st to 5th week sampling time. As mentioned earlier, motility of sperm was also least during these sampling weeks, which indicates that sperm motility and morphology are related to each other. In our results sperm of the treated groups showed tail and head abnormalities. The percentage of abnormal sperms reached closer to the control values in mice treated with all the doses of imatinib during the 10th week sampling time. Maximum sperm abnormalities were seen during the 5th week sampling time in the imatinib treated groups, which indicates that spermatocytes might have been more susceptible to the toxic effect of imatinib. However, the high dose group showed an increased sperm abnormality even in 7th week which indicates that spermatogonia might have also been affected. These findings are supported by our comet assay results which indicated that imatinib caused some damages to the sperm DNA during the 5th week of sampling time. Testicular estrogen acting on estrogen receptor alpha in the efferent ductules is essential for normal fluid reabsorption in the efferent ductules and thus, for normal sperm maturation (28). It is possible that imatinib might have interfered with this process. Hence this may indicate that the fertility index of human subjects taking this drug for cancer treatment may be low and caution should be exercised so that the reproductive capacity of young cancer patients is not affected while they are on treatment with this drug.

Conclusion

Imatinib does affect the sperm parameters of mice significantly, but this effect is reversible once the drug is withdrawn. Imatinib had demonstrated high levels of efficacy in gastrointestinal tumors and also chronic myeloid leukemia. This finding may help the clinicians to plan and address the fertility related issues in young patients of reproductive age who are being treated with imatinib for gastrointestinal tumors and chronic myeloid leukemia.

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