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A peripherally restricted $P2Y_{12}$ receptor antagonist altered rat tumor incidences with no human relevance: Mode of action consistent with dopamine agonism

David A. Brott[a,*], Håkan A.S. Andersson[b,1], Jane Stewart[c], Lorna Ewart[c,1], Greg Christoph[a], Johannes Harleman[c,2], Duncan Armstrong[c], Lewis B. Kinter[a,3]

[a] Drug Safety & Metabolism, AstraZeneca Pharmaceuticals, R&D, Wilmington, DE, USA
[b] Drug Safety & Metabolism, AstraZeneca, R&D, Södertälje, Sweden
[c] Drug Safety & Metabolism, AstraZeneca Pharmaceuticals, R&D, Alderley Park, UK

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ABSTRACT

Background: Ticagrelor is an orally available, direct acting and reversible $P2Y_{12}$ receptor antagonist approved for treatment of acute coronary syndrome. The objectives of these studies were to (1) evaluate the Ticagrelor 2-year rat carcinogenicity bioassay data; (2) investigate potential mode of action (MOA) and (3) interpret human relevance.
Methods: The following studies were done (1) rat two-year carcinogenicity study in male and female rats, (2) *in vitro* and *in vivo* genotoxicity assays, (3) quantitative whole body autoradiography (QWBA; male and female rats), (4) *in vitro* pharmacological profiling for more than 300 assays, and (5) *in vivo* ovariectomized rat assay.
Results: The carcinogenicity study indicated Ticagrelor increased uterine tumor incidence while decreasing mammary and pituitary tumors/hyperplasia incidences in only high dose female rats. However, this altered tumor incidences were not $P2Y_{12}$ target related since marketed non-reversible $P2Y_{12}$ receptor antagonists were not associated with alter tumor incidences. MOA studies determined Ticagrelor exposure in the anterior pituitary and Ticagrelor was (1) non-genotoxic, (2) peripherally-restricted, (3) a dopamine transport (DAT) inhibitor with an IC_{50} lower than systemic free exposure in the rat carcinogenic study and more than a log higher than the free systemic exposure seen in clinical trials and (4) an inhibitor of estradiol-induced prolactin secretion.

Abbreviations: ADP, adenosine-5′-diphosphate; AUC, area under the curve; CHO, Chinese hamster ovary; C_{max}, maximal concentration, DAT, dopamine transport; E_2, estradiol; GLP, good laboratory practice; H&E, hematoxylin and eosin; IC_{50}, inhibitory concentration fifty percent; K_i, inhibition concentration; LC-MS/MS, liquid chromatography-mass spectrometric; LLOQ, lower limits of quantification; MOA, mode of action; MTD, maximum tolerated dose; N_H, Hill coefficient; QWBA, quantitative whole body autoradiography; TK, toxicokinetics.

* Corresponding author. Present address: Enabling Safety Science in Global Regulatory Affairs, Patient Safety and Quality Assurance, AstraZeneca Pharmaceuticals, Wilmington, DE, USA. Tel.: +1 302 893 3131.
E-mail address: david.brott@astrazeneca.com (D.A. Brott).
[1] Present address: Drug Safety & Metabolism, Innovative Medicines and Early Development, AstraZeneca, Mölndal, Sweden.
[2] Present address: Global Preclinical Development & Management, Fresenius-Kabi Deutschland GmbH, Germany.
[3] Present address: Green Lawn Professional Scientific Consulting, LLC, Unionville, PA, USA.

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Similar to Ticagrelor, centrally active dopamine agonists induce the same altered tumor incidence patterns that according to literature do not translate into the clinical setting, with a MOA involving decreased prolactin secretion. The Ticagrelor MOA data and literature suggest that altered dopamine levels in the hypophyseal part of the hypothalamus–hypophyseal axis (by Ticagrelor) will result in similar altered tumor incidences in rat that do not translate into the clinical setting, based on qualitative species differences. In conclusion Ticagrelor increased uterine tumors in the rat carcinogenesis study by a MOA consistent with reduced dopamine inhibition of prolactin, which is not a patient safety risk.

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1. Introduction

Ticagrelor (AZD6140; brand names Brilique™ and Brilinta™, AstraZeneca) is an orally available, direct acting, competitive and reversible $P2Y_{12}$ receptor antagonist, which has therapeutic utility as an oral antiplatelet agent for treatment of acute coronary syndrome and potentially other conditions [1]. The risk of ischemic events is high after acute coronary syndrome and so inhibition of platelet aggregation is a major strategy for preventing ischemia in these patients [51]. Platelet aggregation is a complex process involving many factors, but a major mediator of aggregation is the release of adenosine-5′-diphosphate (ADP) from activated platelets leading to sustained activation of the $P2Y_{12}$ receptor [52,53]. The $P2Y_{12}$ receptor antagonist activity was demonstrated by Ticagrelor (100 mg b.i.d.) inhibiting platelet aggregation by greater than 90% at 4, 12 and 24 h, in humans [54].

The $P2Y_{12}$ receptor is expressed by platelets, brain, vascular smooth muscle cells, dendritic cells and other blood cells [2,3] and is the molecular target of various antiplatelet drugs such as Ticagrelor and the irreversible $P2Y_{12}$ antagonists Clopidogrel and Prasugrel [4,5]. Expression in the brain is equally abundant in the amygdala, caudate nucleus, corpus callosum, hippocampus, substantia nigra and thalamus which are consistent with microglial expression pattern [3]. $P2Y_{12}$ mRNA transcripts were detected [6], but receptor expression was not detected in the anterior pituitary cells [7].

Currently most compounds to which patients will be exposed for more than 6 months duration must be evaluated for carcinogenicity potential during drug development [8]. The two-year rat carcinogenicity bioassay, as outlined in the International Conference on Harmonization guidance documents (ICH S1, S2, S3), is used in conjunction with other assays to determine the carcinogenicity potential of compounds. Human patient safety risk (if any) is determined based on the human relevance framework [9–11]. This framework leverages two concepts to determine a statement of confidence regarding patient safety risk: (1) is the weight of evidence sufficient to establish the mode of action (MOA) in animals and (2) is the MOA plausible in humans. Therefore, determining the MOA of a carcinogenicity finding is critical to accurately determine the human relevance of any findings from the carcinogenicity bioassays. The human relevance framework helps classify the human patient safety risk from high confidence in the rodent carcinogenicity data translating into patient safety risk, to the mechanism of action studies determining the rat carcinogenicity data has a MOA not plausible in human and thereby no patient safety risk.

For example, central-acting dopamine agonists altered tumor incidences in rats is an example of lack of confidence in the MOA translating into human (Fig. 1). This is because altered brain dopamine levels inhibit pituitary prolactin release in both female rats and humans but the decreased prolactin level alters tumor incidences of reproductive organs in female rats and not in humans as prolactin is luteotrophic in rats, but not in primates [35]. Boobis et al. [9] termed this lack of confidence as being due to qualitative species differences.

Therefore, the objectives of these studies were to (1) evaluate the Ticagrelor rat two-year carcinogenicity bioassay data, (2) investigate potential mode of action (MOA) for any altered tumor findings and (3) interpret the data using the human relevance framework to determine the patient safety risk.

2. Materials and methods

All procedures were approved by the appropriate institutional Animal Care and Use Committee (IACUC) in accordance with *The Guide for the Care and Use of Laboratory Animals*. Rats were housed, as outlined within each experiment, with food and water provided *ad libitum*, unless otherwise stated. A standard light–dark cycle was maintained with a timer-regulated light period from 0600 to 1800 h.

2.1. Carcinogenicity study in rats

The procedures within this study were consistent with the guidelines of the EU, US FDA and Japanese MHLW; prospective FDA protocol concurrence was sought and received under the Special Protocols procedure [8].

Briefly, seven-week old Wistar rats (Rat/Wistar Han IGS (Crl: WI(Glx/BRL/Han)GSBR)) in groups of 50 per sex were treated with 0, 20, 60, or 180/120 mg/kg/day Ticagrelor by oral gavage in a 2-year carcinogenicity study. High dose female rats were treated with 180 mg/kg/day and male rats with 120 mg/kg/day. In male rats, the 120 mg/kg/day was selected based on a prior 26-week rat study wherein increased stomach weight and decreased body weight gain in male rats treated with 180 mg/kg/day (data not shown) was deemed above a maximum tolerated dose (MTD)

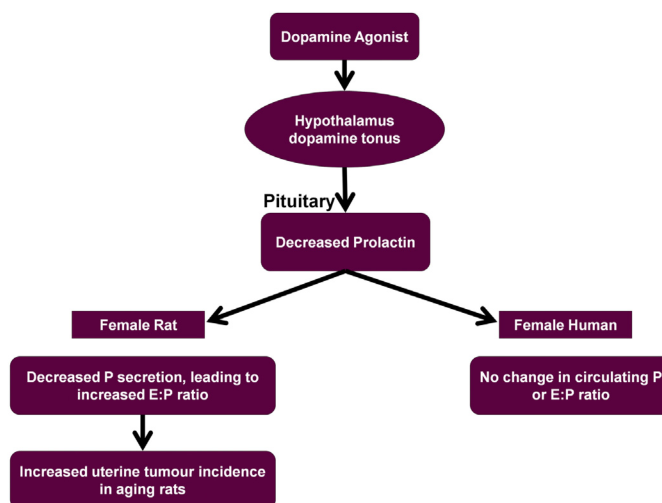

Fig. 1. Mechanisms of action schematic for carcinogenicity in female rats and lack of translation into human (P = progesterone, E = estradiol in rat, estrogen in human).

consistent with unacceptable morbidity/mortality over a 2-year exposure duration. Additional satellite rats were treated with 0 (n = 5/sex), 20, 60 or 180/120 mg/kg/day Ticagrelor (n = 10/sex/dose) for 52 weeks for toxicokinetics (TK) bioanalysis. Ticagrelor was suspended in 1% carboxymethylcellulose with 0.1% polysorbate 80 (w/v, vehicle). The dosing volume was 5 mL/kg with the control (0 mg/kg/day) group receiving vehicle only. The rats were group housed by gender, 5 per home cage. All main study animals were examined macroscopically and microscopically with a full tissue list collected. The tissues were trimmed, embedded in paraffin wax and stained with hematoxylin and eosin (H&E). All slides were examined microscopically and the findings peer reviewed.

On Days 1, 3 and during Weeks 26 and 52, 0.3 mL of blood was collected from the satellite rats at 4 h post dose for 0 mg/kg/day rats and at 2, 4, 6, 8, 12 and 24 h post dose (n = 3 rats/sex/time point) for TK bioanalysis. The blood was collected in 0.5 mL microtainer tubes containing lithium heparin (Becton Dickenson, Franklin Lakes, NJ) and TK bioanalysis of exposure determined by protein precipitation and liquid chromatography followed by mass spectrometric detection (LC-MS/MS).

Rats were fed rodent chow (Lab Diet, Gray Summit, MO) and consumption was measured and recorded weekly up to the end of Week 13 for each cage (n = 5 rats). Between Weeks 14 and 28, food consumption was measured and recorded over approximately one week in every two weeks. After Week 28 food consumption was measured and recorded for one week in every four weeks until the end of the study.

The daily mean food consumption was calculated per rat per day for each period of recording from the total food or water consumption in each cage divided by the number of rats in the cage.

Body weights were recorded once pretreatment, daily for the first 13 weeks of the study and then weekly until end of the study. Any rat showing weight loss or deterioration in condition was weighed more frequently, as necessary.

Statistical analysis of the data were as follows: (1) histological data using Fishers Exact Test (two-tailed), (2) tumor data using SAS (v8.2) PORC MULTITEST at the 5% significance level, and (3) body weight and food consumption of the main study rats were analyzed using Hartley's jackknifed F-max test and Fishers' F-protected t-test.

2.2. Mechanism of action studies

2.2.1. Genotoxicity

Good Laboratory Practice (GLP) genotoxicity assays were carried out using standard methods and materials: Ames *in vitro* assays, Ames *in vitro* assay with metabolite and impurities, mouse lymphoma *in vitro* assay, and rat micronucleus *in vivo* assay [12–15].

2.2.2. Quantitative whole body autoradiography (QWBA)

Male rats or pregnant female rats (Day 18 of gestation) were treated with 3 mg/kg [^{3}H] Ticagrelor (111.4 MBq/mg). Rats were humanely euthanized with carbon dioxide at the designated times post-dose. Immediately prior to euthanizing the rat, a whole blood sample (0.5 mL) was collected into heparinized tubes by venesection of a tail vein and aliquots removed for blood radioactivity analysis. Each rat was immediately frozen and embedded in a block of methyl cellulose. Sagittal sections (30 μm) were prepared, freeze-dried and applied to phosphor screens along with a series of calibration standards containing known concentrations of radioactivity. After 7 days of exposure, the radioactivity present in various organs and tissues were determined using the Cyclone Storage Phosphor system (Packard; Meriden, CT).

Blood sample radioactivity was quantified in scintilant for 5 min, together with representative blank and standard vials using liquid scintillation analyzer with automatic quench correction using an external standard method.

2.2.3. Pharmacological profiling

Ticagrelor and a major active metabolite (AR-C124910) were evaluated at 10 μM in more than 300 receptor,

enzyme and electrophysiological assays (Ricerca Biosciences LLC) including dopamine D_1, D_{2L} and $D_{4.2}$ receptors as well as the dopamine transporter using *in vitro* radioligand binding assays and methodologies described in the literature [16–22]. Human recombinant CHO-K1 cells were used for the dopamine transporter and $D_{4.2}$ receptor, whereas human recombinant CHO cells were used for dopamine D_1 and D_{2L} receptors. The radiolabeled ligands were [^{3}H] SCH-23390, [^{3}H] spiperone [^{3}H] dopamine and [126]RTI-55 for the D_1, D_{2L} and $D_{4.2}$ receptors and dopamine transporter, respectively. The data were calculated as a percentage inhibition of specific binding at the test concentration of 10 μM.

Assays with significant inhibition (>50% effect) at 10 μM were followed up with concentration–response curves. In the case of the dopamine transporter, a concentration–response curve was generated using 10 ascending concentrations in half $\log_{10}$ intervals enabling calculation of the inhibitory concentration fifty percent (IC_{50}), inhibition constant (K_i) and Hill coefficient (N_H). IC_{50} values were determined by a non-linear, least squares regression analysis using the MathIQ^{TM} software (ID Business Solutions Ltd., UK). This software was also used to calculate N_H. The K_i value was calculated using the Cheng–Prusoff equation [23]. These assays were repeated four times in order to generate a robust estimate of affinity.

2.2.4. Rat ovariectomized mode of action study

The rat ovariectomized *in vivo* assay was a modification of Brott et al. [24], in that Ticagrelor was (1) dosed orally once a day for 4 days and (2) on Day 4, Ticagrelor treatment was considered as $t = 0$ h and the rats dosed with estradiol (2 μg/rat) at $t = 1$ h with blood collected at various time points for prolactin analysis.

Female Han Wistar rats (350–375 g; $n = 20$) were ovariectomized and cannulated at Harlan (Indianapolis, IN). Briefly, rats were anesthetized, ovariectomized and allowed to recover for three to five days. The rats were then re-anesthetized, catheters placed in both jugular and femoral veins and externalized at the nape of the neck, and allowed to recover for 7–14 days prior to study initiation. The jugular vein catheter was used for intravenous estradiol administration, whereas the femoral vein catheter was used for remote blood sampling for prolactin analysis. The day prior to experiment initiation, rats were jacketed, tethered and housed individually in home cages at 23 ± 1 °C.

Ticagrelor (180 mg/kg/day; $n = 20$) or vehicle (1% (w/v) sodium carboxymethylcellulose in 0.1% (w/v) polysorbate 80; $n = 10$), were administered orally ($n = 10$ rats/group). Five hours after Ticagrelor treatment on Day 1, 0.5 mL blood was collected into lithium heparin tubes for TK bioanalysis of exposure determined by protein precipitation and liquid chromatography followed by mass spectrometric detection (LC-MS/MS). On Day 4, rats were treated with Ticagrelor or vehicle 1 h before estradiol (E_2; 2 μg/rat). Blood (0.3 mL) was collected from the femoral vein at the following time points: pre-Ticagrelor dose and 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 h post-Ticagrelor dose. The 1 h blood collection was just prior to E_2 treatment. Blood was transferred into microcentrifuge tubes containing the anti-coagulant lithium heparin, and plasma isolated by centrifugation and then frozen at −80 °C until analyzed. Rats were not handled for blood collection; all samples were collected remotely *via* the implanted catheters (*e.g.* from outside of the home cage). Plasma prolactin levels were evaluated by ELISA, according to the Manufacturer's instructions (Kamaya Biomedical Company, Seattle WA; catalog KT-203), except a lower standard was inserted into the assay bringing the lower limits of quantification (LLOQ) down to 1.3 ng/mL. This 1.3 ng/mL LLOQ was deemed acceptable because it was above the mean plus two times the standard deviation of 20 assay diluent samples. The intra- and inter-assay variability were <10%. Several measurements of prolactin were at or below the LLOQ, which was reported as the LLOQ value. Area under the curve (AUC) value for prolactin was calculated for each rat using the Trapezoidal Rule, with data starting from 1 h after Ticagrelor dose, which was just before estradiol dosing, to 5 h post-Ticagrelor dose, collected at 30 min intervals. For the purpose of AUC calculation, the 1 h time point was treated as time point zero. The LLOQ was treated as the baseline (or zero prolactin) value and was subtracted from all prolactin values prior to AUC calculation, to express AUC values relative to the baseline. The mean values were log transformed to stabilize the variance prior to analysis. A *t*-test was then performed on these log-transformed AUC values. Statistical analysis was not performed on the data at each individual time point.

3. Results

3.1. Rat carcinogenicity bioassay

The two year rat carcinogenicity bioassay evaluated Ticagrelor at 0, 20, 60 and 180/120 mg/kg/day with female high dose being 180 and male high dose being 120 mg/kg/day. The AUC exposure of Ticagrelor in high dose female rats (Table 1) remained relatively consistent between Day 1, Week 26 and Week 52, whereas exposure of the metabolite increased between Day 1 and Week 26 and then was similar between Week 26 and Week 52. At 60 mg/kg/day male rats had lower Ticagrelor exposure and higher metabolite exposure, compared to female rats.

Microscopic examination of the tissues revealed that the high dose treated female rats (180 mg/kg/day) had a statistically significantly increased incidence of uterine adenocarcinomas ($p < 0.001$), while there were statistically significantly decreased incidences of tumors/hyperplasia in the pituitary ($p < 0.05$), and mammary ($p < 0.05$) glands (Table 2).

The treatment related effect in the high dose rats (180 mg/kg/day) on the incidence of mammary tumors (decreased) and uterine tumors (increased) are shown in Fig. 2. The coincidence between mammary and uterine tumors showed an inverse relationship in that the rats with a uterine tumor did not have mammary tumors and the rats with mammary tumors did not have a uterine tumor.

Male and female rats in the control and Ticagrelor groups gained body weight throughout the study but the male Ticagrelor-treated rats gained less body weight than the controls over the study period in a dose trend, with the high dose group weighing within 10% of the control group

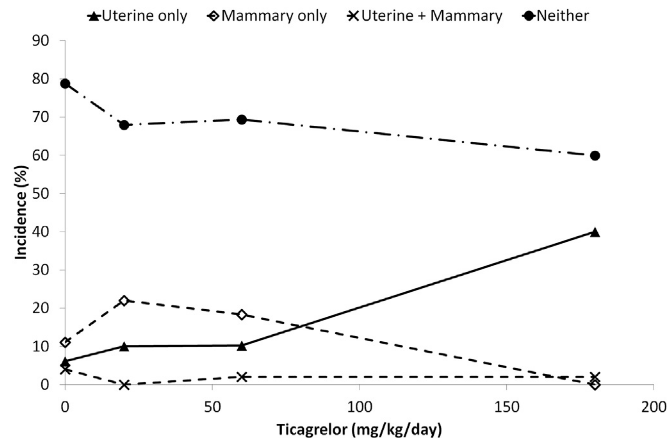

Fig. 2. Uterine and mammary tumor incidences and co-incidences in rat 2 year carcinogenicity study. Rats were dosed daily per gavage for 2 years, necropsied, and tissues collected, trimmed, embedded in paraffin wax, stained with hematoxylin and eosin and microscopically examined with peer review. The number of rats per group were 99, 50, 49 and 50 for 0, 20, 60 and 180 mg/kg/day Ticagrelor.

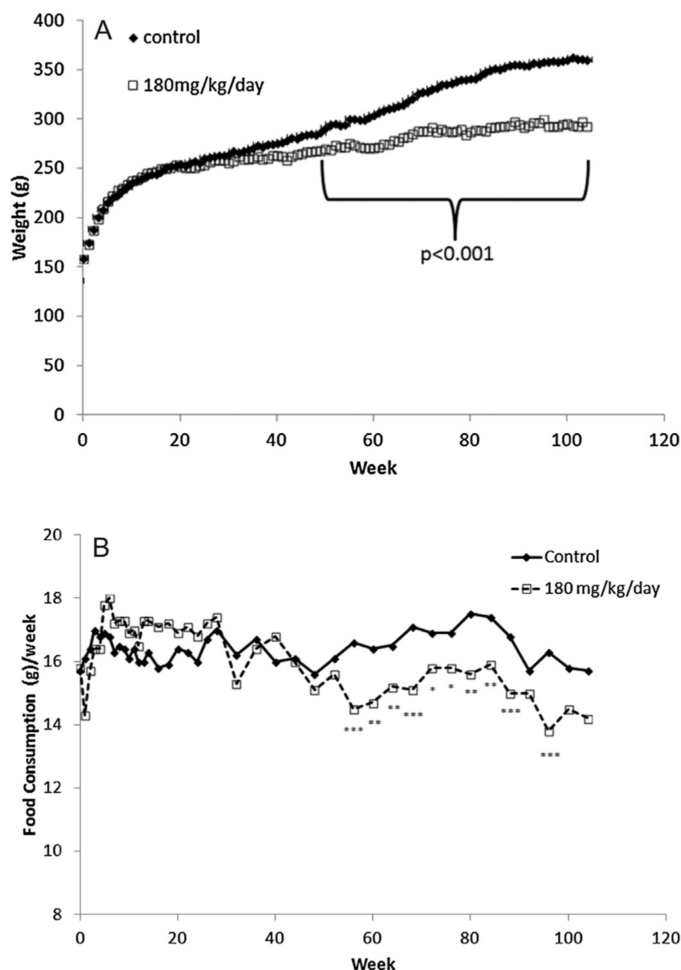

Fig. 3. Body weight and food consumption in rat 2 year carcinogenicity study (female rat). Rats were dosed daily per gavage for 2 years with (A) body weights taken daily from pretrial through Week 13 and then weekly until end of study and (B) food consumption recorded weekly through Week 13, every other week between Weeks 14 and 28 and once every four weeks from Week 29 to end of study. The numbers of rats per group were 99, and 50 for 0 and 180 mg/kg/day Ticagrelor. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 1
Plasma exposure in 2 year rat carcinogenicity study ($n = 5$ rats/group).

Sex	Analyte	Treatment AZD6140 (mg/kg/day)	AUC (*h* μmol/L; mean)		
			Day 1	Week 26	Week 52
Female	Ticagrelor	20	21.1	23.2	31.3
Female	Ticagrelor	60	191	131	163
Female	Ticagrelor	180	545	589	595
Female	Metabolite	20	5.4	7.2	9.3
Female	Metabolite	60	30.8	30.5	39.4
Female	Metabolite	180	66.4	195	195
Male	Ticagrelor	20	11.9	16.8	24.7
Male	Ticagrelor	60	85.5	98.8	129
Male	Ticagrelor	120	200	222	311
Male	Metabolite	20	6.9	16.3	20.0
Male	Metabolite	60	45.8	77.6	102.0
Male	Metabolite	120	93.1	211	257

Table 2
Endocrine-related tumor findings (percentage) in rat 2-year carcinogenicity bioassay ($n = 100$, 50, 49 and 50 female rats for control, 20, 60 and 180 mg/kg/day Ticagrelor).

	0 mg/kg/day	20 mg/kg/day	60 mg/kg/day	180 mg/kg/day
Uterine adenocarcinoma	10	10	12	42***
Uterine adenosquamous carcinoma	0	0	2	4
Uterine squamous cell carcinoma	0	0	0	2
Mammary fibroadenoma	14	22	16	2*
Pituitary hyperplasia and adenoma	66	64	73	50*
Pituitary anterior lobe adenoma in decedent rats	64	55	53	35*

* $p < 0.05$.
*** $p < 0.001$.

at the end of the study. The body weights of the Ticagrelor low and mid dose treated female rats were similar to the control group (data not shown), but the body weights of the high dose treated (180 mg/kg/day) female rats were significantly less ($p < 0.001$) than the control rats, starting at approximately Week 50 through to the end of study and were approximately 20% lower than the control group by the end of study (Fig. 3A).

There were no consistent food consumption differences with Ticagrelor treatment in male rats but in female rats treated with high dose Ticagrelor (180 mg/kg/day) there was increased food consumption early during the study and then significantly decreased food consumption in 10 out of the last 14 measurements (Fig. 3B; $p < 0.05$), such that the decreased food intake starting at Week 52 (food intake measured every 4 weeks after Week 28) corresponded with the decreased body weight gain starting at Week 50.

3.2. *Mode of action studies*

3.2.1. *Genotoxicity*

The Ames, mouse lymphoma and micronucleus assays for Ticagrelor, and Ames and mouse lymphoma assays for major metabolites were negative (Table 3). Further, no regulatory authority has commented that Ticagrelor represents a genotoxicity risk [25].

3.2.2. *QWBA*

Although there were differences in timings of maximal exposures, high levels of radioactivity were observed in adrenal, heart, kidney, liver, pancreas, pituitary, salivary gland, stomach wall and the thyroid (Table 4). Intermediate levels were observed in the skeletal muscles, spleen, thymus and placenta, whereas minimal levels (<0.25 fold of blood levels) were in brain, spinal cord and fetus. Thus, Ticagrelor was deemed effectively excluded from the brain and spinal tissues by the blood brain barrier.

3.2.3. *Pharmacological profile*

Ticagrelor and its metabolite (main circulating metabolite of Ticagrelor and active at the $P2Y_{12}$ receptor) were evaluated for activity at more than 300 secondary targets using *in vitro* radioligand binding, enzyme, and electrophysiological assays. When tested at a single concentration of 10 μM, neither Ticagrelor nor metabolite caused inhibition of radioligand binding at the D_1, D_{2L} and $D_{4.2}$ receptors.

Ticagrelor displaced [^{125}I] Iometopane (RTI-55) from the human dopamine transporter recombinantly expressed in Chinese hamster ovary (CHO) cells, with a pK_i value of 6.79 ± 0.05 (0.202 μM, mean ± standard deviation, $n = 4$ separate experiments; Fig. 4A). The rat free systemic exposure maximal concentration (C_{max}) in the high dose group of 0.502 μM (based on 99.0% protein binding) was above the Ticagrelor IC_{50} of DAT, but rat free systemic exposure in the mid and low dose group C_{max} values of 0.157 and 0.043 μM were below the Ticagrelor IC_{50} of DAT. The human free systemic C_{max} in clinical studies of 0.012 μM (based on 99.2% protein binding) was more than one log below the Ticagrelor IC_{50} of DAT.

The metabolite inhibited radioligand binding at the dopamine transporter with a pK_i value of 6.12 ± 0.08 (0.8 μM, mean ± standard deviation, $n = 4$; Fig. 4B). The rat and human free systemic C_{max} values were more than one log below the metabolite IC_{50} of DAT.

Table 3
Non-clinical genotoxicity study results and conclusion.

Study type	Sex/strain	Dose concentration	Dose, mg/kg	Results	C_{max}, μg/mL	AUC, μg h/mL	Conclusion	Reference
Ames *in vitro* assay	TA1535 TA100 TA102 TA98 TA1537	3–3000 μg/plate	Na	No increased frequency of reverse mutations with or without metabolic activation	Na	Na	Negative	[47]
Mouse lymphoma *in vitro*	L5178Y/TK + 1 mouse lymphoma cells	15.7–41.8 μg/mL (4 h + S9) 5.23–15.7 μg/mL (4 h – S9) 15.7–36.6 μt/mL (24 h – S9)	Na	No increased number of clones associated with treatment	Na	Na	Negative	[48]
Rat micronucleus *In vivo*	Alderly Park (Wistar derived) M&F	Na	2000	No increased frequency of micronuclei at 24 or 48 h post-dose	19 = 24 (M) 45–53 (F)	794–1240 (M) 1140–1360 (F)	Negative	[49,50]

Na – Not applicable.

Table 4
Quantitative whole body autoradiography.

	5 min	0.5 h	2 h	4 h
Male				
Adrenal gland	11.35/5.33[a]	5.26/5.16	1.19/2.57	0.81/2.24
Brain	0.05/0.02	0.04/0.04	0.02/0.04	0.02/0.06
Brown fat	1.35/0.63	0.95/0.93	0.46/0.99	0.52/1.44
Eye	0.50/0.23	0.04/0.04	0.01/0.02	0.14/0.39
Heart	9.15/4.29	4.94/4.84	1.73/3.74	1.18 3.27
Kidney	10.62/4.98	6.77/6.64	2.22/4.79	1.74/4.87
Liver	16.11/7.56	8.44/8.27	3.20/6.97	2.42/6.70
Lung	7.31/3.43	3.90/3.82	2.09/4.51	1.55/4.29
Pancreas	7.35/3.45	7.83/7.68	2.78/6.00	2.08/5.76
Pituitary gland	8.03/3.77	7.03/6.89	1.84/3.97	1.65/4.57
Salivary gland	3.66/1.72	5.57/5.46	2.14/4.62	1.59/4.40
Skeletal muscle	3.15/1.48	3.26/3.20	1.48/3.20	1.16/3.21
Spinal cord	0.05/0.02	0.04/0.04	0.02/0.04	0.02/0.06
Spleen	4.86/2.28	3.81/3.74	1.51/3.29	0.82/2.27
Stomach wall	7.11/3.34	7.17/7.03	2.31/4.99	1.32/3.66
Thymus	0.99/0.46	1.28/1.25	1.37/2.96	1.33/3.68
Thyroid gland	7.84/3.68	3.85/3.77	2.60/5.62	1.19/3.30
Blood (LSC)	2.131	1.02	0.463	0.361
Female				
Fetus	0.03/0.01	0.06/0.07	0.07/0.25	0.03/0.13
Placenta	6.42/2.59	2.86/3.31	1.02/3.62	0.39/1.63
Blood (LSC)	2.474	0.864	0.282	0.24

[a] Raw values of μg equivalent/g/fold of blood value.

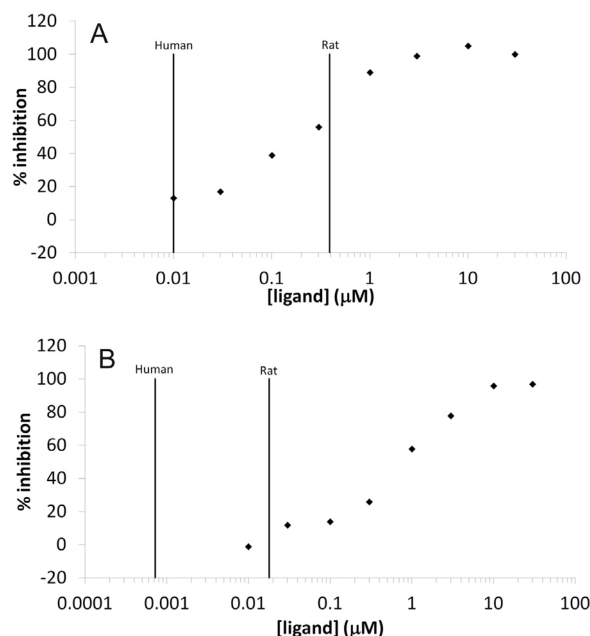

Fig. 4. Inhibition of dopamine transporter (DAT) by (A) Ticagrelor and (B) metabolite. Ticagrelor had substantial inhibition of the dopamine transporter in rats (>50%) and minimal inhibition in humans at free systemic C_{max} levels. The metabolite had no inhibition of the dopamine transporter in rats or humans at the observed free systemic C_{max} levels observed in the corresponding specie.

3.2.4. Investigational rat ovariectomized study

Ticagrelor treated ovariectomized rats were treated for four days with Ticagrelor and then stimulated with estradiol on Day 4 of treatment. Exposure of Ticagrelor and metabolite on Day 1 of dosing were similar to Day 1 and Week 26 exposure in the carcinogenicity bioassay (Table 5). Vehicle control treated rats with estradiol-stimulation had increased prolactin plasma levels between 3 and 4.5 h post vehicle treatment and an AUC of 25.24 ± 18.62 (mean ± standard deviation) (Fig. 5). At 180 mg/kg/day the peripherally-restricted Ticagrelor all but completely blocked the estradiol-induced prolactin release, with an AUC of 9.7 ± 5.53, which was significantly different from the control group ($p < 0.01$). Based upon these findings, Ticagrelor was deemed an inhibitor of estrogen-stimulated prolactin release in the female rat, at the dosage tested.

4. Discussion

The objective of these studies were to (1) evaluate Ticagrelor in the rat two-year carcinogenicity bioassay, (2) investigate potential MOA for any test-article treatment associated tumor findings and (3) interpret the carcinogenicity bioassay and MoA study findings using the human

Table 5
Plasma exposure (5 h post dose) in the ovariectomized rat study compared to carcinogenicity bioassay ($n = 10$ and 5/group, respectively).

Analyte	Treatment (mg/kg/day)	Exposure (μM; mean ± SD)		
		Ovariectomized study Day 1	Carcinogenicity Day 1	Bioassay Week 26
Ticagrelor	180	33.2 ± 10.6	35.9 ± 17.9	37.9 ± 10.5
Metabolite	180	8.1 ± 2.7	3.9 ± 1.4	10.0 ± 4.4

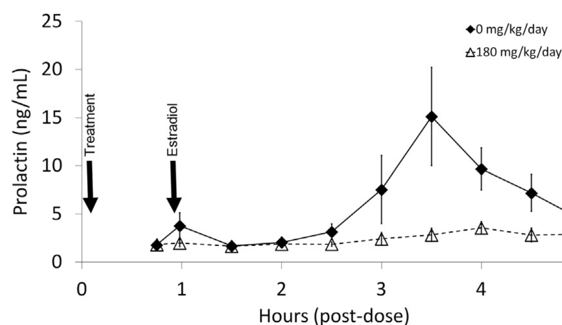

Fig. 5. Ticagrelor inhibits estradiol-induced prolactin release. Dual cannulated and ovariectomized rats were dosed 4 days with 0 or 180 mg/kg/day Ticagrelor. On Day 4, Ticagrelor was $t = 0$ h. Estradiol (2 μg/rat) was dosed at $t = 1$ h with blood collected at multiple time-points and evaluated for prolactin levels. The area under the curve (AUC) values were significantly different ($p < 0.05$), $n = 10$ rats/group.

relevance framework to determine the patient safety risk of Ticagrelor, a $P2Y_{12}$ receptor antagonist.

In summary, in the rat carcinogenicity bioassay, Ticagrelor increased the incidence of uterine tumors and decreased the incidence of mammary and pituitary tumors in the high dose female group; there were no other treatment-associated tumors in any of the treatment groups. The first concept of the human relevance framework is to determine if the weight of evidence is sufficient to establish a MOA in animals. The findings could be due to Ticagrelor being carcinogenic or due to some epigenetic MOA. It was anticipated that Ticagrelor $P2Y_{12}$ receptor antagonism, would not be linked with target related carcinogenicity because marketed irreversible $P2Y_{12}$ antagonists such as Clopidogrel or Prasugrel, did not alter tumor incidences in their respective 2 year carcinogenicity bioassays [4,5]. Therefore, a non-$P2Y_{12}$ mediated mode of action needed to be identified in order to understand the potential translational relevance of the tumor incidences found in female rats. Ticagrelor was also not associated with chemical/structural related carcinogenicity as the genotoxicity studies were uniformly negative for Ticagrelor and major metabolite, and affirmed by all regulatory authorities to date; thus the MoA for treatment-related tumors in female rats is not related to $P2Y_{12}$ receptor antagonism or DNA alterations, but must be the result of an epigenetic mechanism.

The rat carcinogenicity study findings including inverse relationships between incidence of uterine, with mammary and anterior pituitary tumors, and body weight gain effects were consistent with those previously reported for centrally-acting dopaminergic agonists (*i.e.* Bromocriptine) [26] and so the epigenetic MOA hypothesis was that Ticagrelor was carcinogenic in female rats due to altered prolactin drive, possibly *via* the dopaminergic system. Evidence in the current studies supporting this hypothesis included (1) primary and secondary pharmacological testing identifying Ticagrelor binding and inhibiting the dopamine transporter, and (2) Ticagrelor inhibition of estrogen-stimulated prolactin release was confirmed in the ovariectomized estradiol-challenge model, at the dose associated with treatment-related tumor changes in the carcinogenicity bioassay. A difference from centrally-acting dopaminergic agonists was that Ticagrelor was peripherally restricted and would increase dopamine levels in only the pituitary by inhibiting dopamine reuptake (Fig. 1). In the pituitary this effect is possible because of the lack of blood brain barrier in this organ.

In addition to similarities in altered tumor incidences, both centrally-acting dopaminergic agonists and Ticagrelor altered body weight gain. In fact, tumor incidences and body weight gain are closely inter-connected based on dopamine inhibition of prolactin secretion. Prolactin is orexigenic (an appetite stimulant) and induces hyperphagia [27]. The dopamine agonist bromocriptine, which inhibits prolactin release, also inhibited weight gain in Wistar rats [28]. Prolactin is a peptide hormone produced and secreted by lactotrophs in the anterior lobe of the pituitary gland; in the female rat; prolactin is under positive regulation by estradiol and negative regulation by dopamine [29–32]. Estradiol stimulates the synthesis and release of prolactin and can act directly or indirectly to modulate the activity of the dopaminergic system on prolactin release from the pituitary [33]. Dopamine from hypothalamic dopaminergic neurons decreases prolactin release by exerting an inhibiting effect on the lactotrophs *via* the dopamine (D_2) receptor [31]. Dopaminergic compounds known to inhibit estradiol-induced prolactin release [24] such as the centrally-acting D_2 agonist bromocriptine are known to alter tumor incidences in female rats with a profile similar to Ticagrelor [26,34]. More specifically, bromocriptine can induce hypoprolactinemia in rats and humans, but increases uterine and decreases mammary tumors only in rats, which is postulated to be due to a direct prolactin impact on rat ovarian steroidogenesis in aged rats [34,35]. A difference between Ticagrelor and centrally-acting dopamine agonists is that the QWBA data show that Ticagrelor is peripherally restricted and thus not likely to influence dopaminergic mechanisms in the hypothalamic end of the hypothalamic–hypophyseal axis. However, the QWBA study did demonstrate Ticagrelor levels in the pituitary gland, with the anterior pituitary being outside of the blood brain barrier. Other peripherally-restricted compounds such as the dopamine receptor agonist carmoxirole impacting dopaminergic regulation of prolactin release would be active at this hypophyseal end

of the axis [24]. Therefore, it is reasonable to hypothesize that Ticagrelor exerts its effect at the level of the anterior pituitary gland, outside the blood brain barrier and due to peripheral exposure. Alternatively, because the effect only occurs with the highest systemic exposure to Ticagrelor tested in rats we cannot categorically rule out the possibility that the effect is in part attributable to a very small fraction of the Ticagrelor exposure that may penetrate the rat blood brain barrier.

Another difference between Ticagrelor and the dopamine agonists evaluated to date is that Ticagrelor's MoA is inhibition of the dopamine transporter (DAT) and lacks intrinsic dopamine agonist activity. To our knowledge, Ticagrelor is the first peripherally-restricted compound with non-target related DAT activity above the IC_{50} value to undergo a 2-year carcinogenicity bioassay. DAT inhibition will increase endogenous dopamine levels by blocking dopamine reuptake and thereby have similar downstream effects as treatment with dopamine or dopamine agonists.

Ticagrelor free systemic exposures in the carcinogenesis study and the DAT IC_{50} give credence as to why altered tumor incidences were only observed in the high dose rats, since the C_{max} of the high dose females was above the Ticagrelor IC_{50} of DAT while the C_{max} in the mid and low dose groups were below the Ticagrelor IC_{50} of DAT.

In the context of the human relevance framework [9], the similarity of multi-organ carcinogenicity data and body weight gain profiles between Ticagrelor and other dopaminergic compounds is sufficient weight of evidence to establish inhibition of dopamine reuptake and potentiation of endogenous dopamine agonist activity at the level of the anterior pituitary by Ticagrelor as its MOA for the findings in the rat carcinogenicity bioassay. In addition, since Ticagrelor is peripherally restricted it is likely that this inhibition of dopamine transport and potentiation of endogenous dopamine occurs at the level of the lactotrophs in the pituitary, thus peripheral and not central dopamine levels are most likely responsible for the rat carcinogenesis findings.

The human relevance framework helps classify the human patient safety risk from high confidence in the rodent carcinogenicity data translating into patient safety risk, to the mechanism of action studies determining the rat carcinogenicity data has a MOA not plausible in human and thereby no patient safety risk. Three characterized examples of the application of the human relevance framework are:

(1) high confidence in the human relevance of the ethylene oxide rat carcinogenicity data because it was found to be genotoxic in *in vitro* and *in vivo* studies, a mechanism which is not specific to a single species [11],
(2) low confidence in the human relevance of thiamethoxam-related mouse liver tumors because, although the MOA is found in both rodents and humans, the key metabolites associated with the tumors generated in the mouse were not found in sufficient concentration in rat or human [36]. This low confidence in translation is also referred to as quantitative differences in key events between animals and humans [9] and
(3) Lack of confidence in human relevance of centrally active (peripheral and brain exposure) dopamine agonist induced uterine tumors and decreased mammary tumors, *because* the mode of action is nongenotoxic and female rat specific [34,37]. This lack of confidence in translation is also referred to as qualitative differences between animals and humans [9].

Based on the human relevance framework, the next step in evaluating patient safety risk was to determine if the Ticagrelor rat carcinogenicity MOA was plausible in humans. In order to determine this, there was a need to understand both the differences between DAT inhibition in the rat *versus* human as well as how hypoprolactinemia can lead to uterine tumors and if the mechanism is similar in humans.

In normal reproductive cycling rats, the estrus cycle consists of 4 days (proestrus, estrus, diestrus-1 and diestrus-2). Prolactin levels are low throughout the estrus cycle except during the afternoon of proestrus, which is driven by the rising estrogen levels in the morning of proestrus [38]. The prolactin released during proestrus is luteotropic in that it promotes rescue of the corpus luteum from degradation, but prolactin is also essential for progesterone production after ovulation, which antagonizes the estradiol-stimulated uterine growth [39]. With aging in rats, there is a progressive loss of hypothalamic dopaminergic neurons, which decreases the level of dopamine in the pituitary and resulting in higher prolactin release [40,41]. The increased prolactin levels are a critical factor in the age related transformation from normal estrus cycling into pseudopregnancy and ultimately leading to anestrus (reproductive senescence) with the rat in a state of progesterone dominance. Therefore, in the rat, age-related anestrus can be a result of decreased dopamine levels. In untreated control rats there is also a negative relationship between the presence of uterine and mammary tumors [42]. The same relationship was observed in the current study. In rats, prolactin is the major stimulating factor for the development of mammary tumors which is closely related to the presence of pituitary hyperplasia or tumor. Animals with a uterine tumor have significantly lower incidence of mammary tumors and *vice versa*, demonstrating the close biological link between these tumor patterns and incidence [42]. Increasing dopamine levels in aging rats will decrease prolactin levels, which cause not only decreased stimulation mammary glands, but also luteolysis, new follicle development and thereby the rat will continue to be exposed to recurrent estradiol. Thus in older female rats, decreased prolactin levels will increase the estradiol:progesterone ratio over a series of cycles (relative estrogen dominance). This prolonged estrogen stimulation of the endometrium can lead to the observed endometrial adenocarcinoma seen with bromocriptine or other compounds that increase dopaminergic stimulation.

Unlike the rat, prolactin is not essential for adequate progesterone production by the corpus luteum in human [43]. The differences in the role of prolactin between rat and human in female reproductive cyclicity are the reasons

why the tumorigenic effects on the uterus of compounds that increase dopamine levels are considered to be rat specific and not relevant to pathophysiological conditions in human, based on qualitative species differences between rat and human.

Epidemiological studies support the rat specific tumorigenic potential of compounds like bromocriptine in that compounds that increased dopamine levels are not associated with increased endometrial adenocarcinomas in women [26,34,44,45].

A potential limitation of these studies includes the lack of hormone (*i.e.* prolactin, progesterone and estradiol) measurements in rats. Hormone levels were not included in the 2-year rat carcinogenicity study since based on other $P2Y_{12}$ antagonists the altered tumor incidences were unexpected findings. An additional study to evaluate Ticagrelor induced hormone changes would have been very difficult for the following reasons. Based on the findings that food-intake and weight gain were not decreased until after 52 weeks of dosing within the carcinogenicity study, thus a study would either have required the use of older female rats (greater than a year of age) or a chronic study of dosing rats for more than a year. As progressive aging of the neuroendocrine system show great inter-individual variation, large group sizes would have been require. Furthermore, hormonal monitoring in cycling females requires staging and multiple sampling, and since prolactin is a stress sensitive hormone this would restrict sampling by indwelling catheters [46]. Therefore, we thought such large, complicated long term studies unnecessary to establish MOA within the rat, based on the unique findings of altered tumor incidences being similar between Ticagrelor and dopaminergic compounds and the supportive finding of the MOA studies.

A second potential limitation of our data includes the lack of hormone (*i.e.* prolactin, progesterone and estrogen) measurements in clinical studies. Based on the qualitative species differences of Ticagrelor and other dopaminergic compounds being post prolactin secretion (Fig. 1), hormone analysis would have been expected to be very important in clinical studies with expected findings being altered prolactin levels without changes in progesterone or estrogen levels. However, based on quantitative species differences, hormone measurement was deemed not appropriate in clinical studies, based on (1) Ticagrelor free systemic exposure in the rat was above the Ticagrelor IC_{50} of DAT that would result in increased prolactin in the rat, but (2) Ticagrelor free systemic exposure in humans was below the Ticagrelor IC_{50} of DAT and so prolactin increase due to DAT inhibition would not be expected to be observed in the clinical setting and thus the rationale as to why hormone levels were not evaluated in clinical studies. Therefore qualitative species differences explain why the rat tumor findings pose no human safety risk, while quantitative species differences explain the rat tumor findings (DAT inhibition above IC_{50} value in high dose treated rats and below IC_{50} in mid and low dose rats) and why hormone analysis in clinical studies was not appropriate.

In summary, Ticagrelor an orally available, direct acting, competitive and reversible $P2Y_{12}$ receptor antagonist increased uterine tumors and decreased mammary and pituitary tumors in the rat 2-year carcinogenicity bioassay. Mode of action studies showed that the mechanism as epigenetic interruption of dopamine regulation of prolactin release from the anterior pituitary gland. The investigational study determined peripherally-restricted compounds that increase dopamine levels can alter tumor incidences with a MoA consistent with those observed for centrally active dopamine agonists, suggesting centrally active dopaminergic compounds could be altering tumor incidences at least partially due to peripheral exposure. This MoA of decreased prolactin release is luteotrophic in rats that with advancing age lead to disturbances in female reproductive organs and increased uterine tumors. Prolactin is not luteotrophic in humans and therefore the rat carcinogenicity data for Ticagrelor do not pose a patient safety risk, based on qualitative species differences between rat and human.

Conflict of interest

The authors have no conflict of interest to declare.

Transparency document

The Transparency document associated with this article can be found in the online version.

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