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Demonstration of Nucleoside Transporter Activity in the Nose-to-Brain Distribution of [^{18}F]Fluorothymidine Using PET Imaging

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Abstract

Purpose—To evaluate the role of nucleoside transporters in the nose-to-brain uptake of [^{18}F]fluorothymidine (FLT), an equilibrative nucleoside transporter (ENT1,2) and concentrative nucleoside transporter (CNT1–3) substrate, using PET to measure local tissue concentrations.

Methods—Anesthetized Sprague-Dawley rats were administered FLT by intranasal (IN) instillation or tail-vein injection (IV). NBMPR (nitrobenzylmercaptopurine riboside), an ENT1 inhibitor, was administered either IN or intraperitoneally (IP). Dynamic PET imaging was performed for up to 40 minutes. A CT was obtained for anatomical co-registration and attenuation correction. Time-activity curves (TACs) were generated for the olfactory bulb (OB) and remaining brain and the area-under-the-curve (AUC) for each TAC was calculated to determine the total tissue exposure of FLT.

Results—FLT concentrations were higher in the OB than in the rest of the brain following IN administration. IP administration of NBMPR resulted in increased OB and brain FLT exposure following both IN and IV administration, suggesting that NBMPR decreases the clearance rate of FLT from the brain. When FLT and NBMPR were co-administered IN, there was a decrease in the OB AUC while an increase in the brain AUC was observed. The decrease in OB exposure was likely the result of inhibition of ENT1 uptake activity in the nose-to-brain transport pathway.

Conclusion—FLT distribution patterns show that nucleoside transporters, including ENT1, play a key role in the distribution of transporter substrates between the nasal cavity and the brain via the OB.

Keywords

intranasal administration; nucleoside transporters; [^{18}F]fluorothymidine; ENT1; CNT3; nose-to-brain transport

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Introduction

Direct material transfer from the nasal cavity to the brain has been reported for a number of drugs and protein-based therapeutics. (1, 2) The transfer of materials directly from the nasal cavity to the brain has been proposed to involve multiple pathways, including transcellular and paracellular transport through the nasal epithelium; transaxonal transport involving the olfactory and trigeminal axons which innervate the nasal mucosa; and perineuronal or perivascular transport via the fluids and spaces immediately adjacent to the olfactory or trigeminal nerves and mucosal vasculature followed by subsequent transfer of the material into the cerebrospinal fluid (3, 4). Each of these pathways can result in the nose-to-brain transfer of materials, and the specific pathway(s) involved for a particular material are likely dependent on multiple parameters including size (molecular, macromolecular, nanoparticulate), solubility, and the substrate characteristics of those materials.

The role of uptake and efflux transporters in the regulation of absorption across the nasal mucosa with the subsequent potential to enhance or limit transfer to the brain has been the subject of recent investigation. (3, 5–10) Uptake transporters in the OCT family have been shown to influence the nose-to-brain transfer of drug substrates (11), and efflux transporters, particularly P-glycoprotein, have been shown to modulate the transfer of drug substrates across the nasal mucosa (6, 7, 10).

The nasal administration of several nucleoside-like drugs, including zidovudine (AZT) and stavudine (d4T), has been investigated in an effort to improve CNS exposure for the treatment of HIV infection. (12, 13) Both nucleoside drug compounds were found in measurable concentrations in the CNS following nasal administration, but an improved distribution into the CNS following intranasal administration compared to IV administration was not able to be demonstrated for either of the compounds. Zidovudine is a substrate for a number of known transporters, including members of the organic cation, organic anion, and multi-drug resistance families. Zidovudine is also a substrate for several nucleoside transporters, including CNT1 (concentrative nucleoside transporter family) and ENT2 (equilibrative nucleoside transporter family). (14) Similarly, stavudine is reported to be a substrate for at least OAT1 and CNT1. (15) It is likely that the complex interactions between these uptake, facilitative, and efflux transporters combine to result in specific distribution patterns for these compounds when administered via the nasal cavity.

In an effort to improve the understanding of the ability of nucleoside transporters present in the nasal mucosa to accomplish enhanced distribution of nucleoside-like drug compounds to the brain, a commonly used PET ligand, [^{18}F]Fluorothymidine, was identified as a promising probe molecule to investigate the transfer of nucleoside transporter substrates via the nasal cavity to the brain. Fluorothymidine, a thymidine analogue, has been investigated as a therapeutic antiviral (alovudine) (16), whereas, [^{18}F]Fluorothymidine (FLT) is a positron-emitting radiopharmaceutical used to map proliferation in tumors and bone marrow. (17) FLT has minimal permeability across the intact blood brain barrier (BBB) (18) and, as a result, is potentially useful in the diagnosis and/or monitoring of brain tumors where there is disruption in the BBB. (19) Its exclusion from the normal brain compartment when

administered systemically makes FLT an excellent probe to investigate CNS distribution resulting from direct transport between the nasal cavity and the brain.

FLT/alovudine is a substrate for both the Equilibrative Nucleoside Transporter family (SLC29), sodium-independent transporter of purine and pyrimidine nucleosides and for a number of nucleobases; and the Concentrative Nucleoside Transporter family (SLC28), sodium-dependent transporters of pyrimidine- and purine-nucleosides and a number of other nucleoside-like molecules. (20) ENT1 and CNT3 have been demonstrated to be expressed in the nasal mucosa and are present primarily on the apical surface of the respiratory and olfactory epithelia in the nasal cavity. (9, 21) Using cell-based techniques, Paproski, et al. demonstrated that alovudine (FLT analog) was a substrate for ENT1, ENT2, CNT1 and CNT3. (22) The equilibrative transporters were also shown to have lower affinities but greater capacities for alovudine transport than the concentrative nucleoside transporters. In the presence of NBMPPR (nitrobenzylmercaptapurine riboside), an ENT1 inhibitor, the uptake of alovudine was decreased by at least 50%, indicating that the ENT1 transporter was acting in concert with other transporters in the absorption of this antiviral compound. Unfortunately, specific inhibitors able to be administered to animals as tools to evaluate CNT, especially CNT3 transporter activity, have yet to be identified. In a follow-up study, Paproski, et al. reported the investigation of the biodistribution of FLT using PET and demonstrated the important role of ENT1 in the distribution of FLT, including its distribution into the brain. (23)

A further advantage of selecting FLT as a model nucleoside to evaluate nasal absorption and subsequent distribution is the power of its compatibility with hybrid imaging technologies such as positron emission tomography (PET) coupled to computed tomography (CT) scanners to provide a means to visualize and quantify the pharmacokinetics of labeled compounds (via PET) co-registered to anatomy (via CT). The ability to use dynamic imaging techniques also enables the investigation of the distribution of a labeled compound in a live, non-surgically modified animal (including humans) which reduces the likelihood of experimental artifacts resulting from alterations in normal physiologic activities due to surgical procedures designed to allow for tissue or biological fluid sampling. Other investigators have utilized PET to evaluate nasal absorption and distribution patterns (24–26), yet none of these reports have focused on the evaluation of the role of epithelial transporters in the enhanced nasal absorption and brain distribution of substrates. The previous imaging results, along with the unique goal of the present work, demonstrate that hybrid imaging techniques are powerful tools to assist in the evaluation of the deposition of drugs and formulations within the nasal cavity (27) along with the determination of the pharmacokinetics and distribution profiles of nasally administered drugs. The selection of FLT, a known nucleoside-transporter substrate, also enabled the evaluation of transporter effects on the absorption and distribution of substrates from the nasal mucosa directly to the brain.

The purpose of this investigation was to determine whether epithelial transporters can modulate the transfer of substrates between the nasal cavity and the brain without requiring uptake into the systemic vasculature or direct interaction with the pathways provided by the olfactory and trigeminal innervation of the nasal mucosa. Specifically, these studies were

focused on the evaluation of the role of the nucleoside transporters, specifically the ENT1 transporter, in the nose-to-brain transport of FLT, an equilibrative and concentrative transporter nucleoside transporter substrate using the power of imaging to enable the quantification of the distribution of the substrate between these regions. Since FLT is a substrate for multiple nucleoside transporters, the investigation of individual transporter contributions was not possible. The significance of ENT1, the nucleoside transporter with highest FLT affinity, was able to be investigated using an ENT1-specific inhibitor, NBMPR, to investigate whether cellular-based transporters play a role in enhancing the transfer of substrates from the nasal cavity to the brain via non-neuronal pathways. The distribution of FLT was evaluated after intranasal and systemic administration of FLT alone and in combination with NBMPR administered either intranasally (IN) or intraperitoneally (IP), to compare brain distribution patterns following intranasal and intravenous delivery of the transporter substrate.

Materials and Methods

Radiopharmaceutical dose preparation

[¹⁸F]Fluorothymidine (FLT) was produced in a manner equivalent to that employed for research human doses as described in IND # 101023 (Dr. Michael Graham, MD, PhD, PI)¹ and then further processed to reduce the approximately 9% ethanol content to less than 1% in order to be safely administered to the animals in this investigation.

Animal preparation and imaging

Anesthetized Sprague-Dawley rats (~250–400 g) were administered FLT by intranasal instillation (IN) (volume = 20 – 25 μ L, range 2.2 to 6.7 MBq) or tail-vein injection (IV) (volume ~ 100 μ L, range 6.2 to 11.1 MBq). Sedation consisted of 50 mg/kg pentobarbital intraperitoneally rather than an inhalation anesthetic (e.g., isoflurane). Although inhalation anesthetic agents are more commonly used in small animal imaging, because of the potential for inhalation agents to alter nasal physiology (e.g., tissue response to gas flows, drying of nasal passages), IP pentobarbital was used instead. In order to minimize the potential for breathing difficulties, IN administration was made to a single nostril only. NBMPR (nitrobenzylmercaptapurine riboside), an ENT1 inhibitor, was administered either IN (1 mM in 10% DMSO; 25 μ L or IP (3 mg suspension in normal saline (600 μ L)) prior to the administration of the FLT (32 $\pm$ 7 minutes) to allow for absorption and distribution of the inhibitor. (28) Both IN and IP inhibitor administration and a “no inhibitor” control were tested for each route (IN or IV) of FLT administration resulting in six different sets of conditions. After instillation (as described in Ponto, et al. (27)) or injection of the radiopharmaceutical, the rat was immediately transferred to the bed of an Inveon Docked PET-SPECT-CT system (Siemens Medical, USA), placed in the supine position with the head at an approximately 40 degree angle to the bed that was equipped with an internal heater. Imaging consisted of a list-mode acquisition for up to 40 minutes. The time between start of administration and start of imaging was accounted for in the quantitation. A CT

¹The University of Iowa's IND for FLT is essentially equivalent to that utilized in the FLT Demonstration Project of the Clinical Trials Network of the Society of Nuclear Medicine and Molecular Imaging (SNMMI CTN). For further information on specifications for FLT, see the Centralized Biomarker INDs, <http://www.snm.org/index.cfm?PageID=8819>

image was obtained for attenuation correction and anatomical co-registration with the PET image. PET data were histogrammed into a dynamic sequence and reconstructed using filtered back-projection and a ramp filter to create a dynamic series of images. All procedures were approved by and conducted in accordance with the policies of the University of Iowa Institutional Animal Care and Use Committee (IACUC).

Image and data analyses

All image analyses were performed using the view, volume-of-interest and co-registration (fusion) tools of the PMOD Biomedical Quantification Package (version 3.3, PMOD Technologies, Ltd., Zurich, Switzerland). Volumes-of-interest (VOIs) for the olfactory bulb (OB) and rest of the brain (brain) were created by manually drawing regions on the inside surface of the skull on all relevant CT slices. The VOIs were digitally transferred to the co-registered dynamic PET images. Time-activity curves (TACs) were generated for the OB and residual brain VOIs and scaled in terms of percent dose administered. The 0 – 40 minute area-under-the-curve (AUC in fraction of dose $\times$ seconds) for each TAC was calculated to determine the total tissue exposure of FLT during the imaging session. The no inhibitor conditions were based on $N = 5$ total measurements (3 rats with replicates in 2 animals) for IN administration and $N = 3$ total measurements (2 rats) for IV administration. One rat had replications for both IN and IV administrations. The inhibitor studies were based on $N = 2$ measurements (2 rats) for each condition. Because of the small number of animals employed and the slightly different sampling and binning protocols for each animal due to the delay between instillation/injection and scanner positioning, error bars were not included on the plots.

Ex vivo analyses

Selected animals were euthanized (pentobarbital, 150 mg/kg) immediately following imaging and the brain was harvested and dissected into anatomical structures (right and left olfactory bulb, right and left hemispheres, cerebellum, medulla oblongata). Each structure was placed in a pre-weighed microcentrifuge tube. Each tube was weighed and counted in a pre-calibrated well counter. The net weight and the total quantity of radioactivity present were determined for each structure. Taking into account the radioactive decay, the percentage of the administered dose at the time of euthanasia was calculated for each structure and for the total OB and balance of the brain (without OB).

Results

Following IN administration without NBMPR, imaging-based FLT concentrations, expressed as percent administered dose per volume (cc) of tissue, were higher in the olfactory bulb than in the residual brain (i.e., brain tissue not including olfactory bulb) (Figure 1).

The IP administration of the nucleoside transporter inhibitor, NBMPR, resulted in higher FLT concentrations (percent administered dose per volume (cc) of tissue) in both the olfactory bulb and the residual brain following either IN (Figure 2A) or IV (Figure 2B) FLT administration. When FLT and NBMPR were co-administered via the IN route, there was a

dramatic decrease in the olfactory bulb concentrations and the resulting AUC was significantly reduced compared to no inhibitor (−58.4% reduction or 41.6% of IN/no NBMPR AUC) or IP inhibitor conditions (32.7% of IN FLT/IP NBMPR)(Figure 2A). Higher concentrations in the olfactory bulb over time were also observed after IV FLT administration with NBMPR, either IP (+23.8%) or IN (+30.8%), than without (Figure 2B) although the IV route for FLT resulted in concentrations approximately 1/20 of those observed after IN administration during the early time periods. The time course of the concentration in brain (in percent administered dose/cc) with and without inhibitor by route of administration are presented in Figure 3. The presence of inhibitor by either the IN or the IP route, increased the concentration of FLT in the brain relative to no inhibitor with IN FLT/IN inhibitor resulting in the highest concentrations.

FLT tissue exposure, as defined by the AUC (from time = 0 to 40 minutes), was higher after IN than IV administration for both the olfactory bulb and the residual brain (Figure 4 (top plot) bars I & IV; Figure 5 (top plot) bars I & IV). IP administration of NBMPR increased the AUC in both the olfactory bulb (Figure 4 bar II) and the residual brain (Figure 5 bar II) after FLT administration via the IN route and, to a lesser extent, following IV administration (Figure 4 bar V; Figure 5 bar V). Co-administration of the inhibitor and FLT by the IN route, drastically decreased the AUC in the olfactory bulb (Figure 4 bar III) but increased the AUC in the brain (Figure 5 bar III). In comparison, IN administration of the inhibitor only slightly increased the AUC in either tissue after IV FLT administration (Figure 4 bar IV & VI; Figure 5 bar IV & VI). Following IN administration, the majority of the FLT appears to clear from the OB within the first 20 minutes (1200 seconds), and a slower clearance phase is responsible for the low and prolonged concentration measured over the remaining imaging period (see Figure 1). Therefore, additional analyses of the AUC patterns for the 0–20 minute and 20–40 minute intervals were conducted to determine whether there were significant differences in the distribution behaviors between these time periods. The results demonstrated that the pattern in the AUC under the various experimental conditions was essentially identical during the first 20 minutes after FLT administration and the subsequent 20 minute imaging interval. (i.e., 0 to 20 minutes and 20 to 40 minutes post-administration) for the brain but differed substantially between these time periods for the OB after IN administration of FLT. These differences reflect the alterations in the effect of the inhibitor by route of administration (IP versus IN) where rapid absorption and clearance of the inhibitor from the nasal mucosa results in diminishing inhibition of ENT1 over time with resulting alterations in local OB concentrations.

Since spillover and partial volume effects can potentially influence the magnitude of imaging-based tissue concentration estimates, ex vivo analyses of the uptake into the OB and brain were performed in select animals. Figure 6 plots the fraction of the administered dose in the last imaging frame (35 to 40 minutes post-administration) compared to the quantity determined by assaying the dissected tissues (OB versus the balance of the brain including cerebellum and medulla). The time difference between the last imaging frame and euthanasia was between 11 and 12.3 minutes with the exception of one of the animals in the IV FLT/IP NBMPR group which had a 44 minute delay. Although the values are not identical between the imaging-based and directly-measured tissue concentrations, the quantities are very similar and exhibit the same pattern between the routes of administration

and the presence and absence of NBMPR. The IV FLT/IP NBMPR condition in the brain experienced the longer time delay (44 min), and this extended time period may have resulted in greater washout from the brain than for the other samples.

Discussion

The results of these studies clearly demonstrate that the distribution of FLT (no inhibitor) into the brain is quite different following intranasal administration as compared to intravenous administration. As expected, the brain (and olfactory bulb) exposure to FLT was limited following intravenous administration due to its poor BBB permeability. Over the time course of imaging, 0.09% or less of the administered dose was measured in the total brain (including olfactory bulb) at any given time following IV administration. In comparison, the total brain FLT content following intranasal administration ranged from 0.3% early post-instillation to 0.09% at later times, a value similar to what was observed after IV administration. Based on the proposed pathways for nose-to-brain distribution(4), it was also anticipated that the olfactory bulb concentrations would be significantly higher following intranasal administration compared to intravenous administration. The olfactory bulb concentration values measured following intranasal administration (Figure 2A) were much higher than those following systemic administration (Figure 2B) and the remaining brain exposures paralleled the observations from the olfactory bulb (Figure 3). These measurements clearly demonstrate that some fraction of the intranasally administered dose of FLT transfers directly to the brain without relying on systemic absorption with subsequent transfer across the BBB. For all of the IV FLT administration combinations, the olfactory bulb and remaining brain FLT concentrations were quite low. This confirms that FLT has limited permeability across the BBB, and the significantly increased FLT concentrations in the brain following intranasal administration, even when NBMPR was included, clearly demonstrates the importance of the direct nose-to-brain pathways in FLT distribution.

While the direct nose-to-brain transfer of FLT is an important finding, PET imaging is not sufficiently sensitive to enable the visualization of the pathway utilized by FLT to enter the olfactory bulb and more distant brain tissues. Since FLT is known to be an ENT1 substrate, the role of the nucleoside transporters present in the nasal epithelial cells contributing to the transcellular absorption pathway was probed by administering an ENT1 inhibitor, NBMPR, along with FLT. Since Paproski, et al. demonstrated a greater than 50% reduction in alovudine uptake in the presence of NBMPR in a cell-based assay (23), it is likely that that inhibition of only ENT1 in the rat should still result in a measurable change in uptake if the epithelial transporter pathway is important for FLT absorption in the nasal mucosa. When the ENT1 inhibitor, NBMPR, was administered IP, both the olfactory bulb and remaining brain AUCs were increased following either intranasal or intravenous FLT administration. This suggests that inhibitor (NBMPR) concentrations in the systemic circulation were able to inhibit ENT1 activity in the brain microvasculature, choroid plexus, or parenchyma which resulted in a reduction in the clearance of FLT from the brain. Further observation that the changes in FLT distribution following systemic administration were similar for both IP and IN administered NBMPR (Figure 5 bar V & VI) also suggests that the inhibitor is absorbed from both the nasal and peritoneal cavities into the systemic circulation. The decrease in FLT distribution into the olfactory bulb following inhibition of ENT1 activity by NBMPR

administered intranasally demonstrates the importance of ENT1 in the uptake of FLT by the nasal mucosa.

The power of hybrid imaging techniques and the availability of a unique substrate compound, FLT, have enabled the demonstration of the role of epithelial transporters in the enhanced distribution of drugs into the brain following intranasal administration. Even with the availability of an excellent probe substrate, however, the results of these and previous studies underscore the complexity of drug absorption and distribution processes. For example, FLT is reported to be a substrate for at least 4 nucleoside transporters, two of which are known to be expressed in the nasal mucosa. (21, 25) Based on the reported substrate interactions of other nucleoside-like drug compounds, additional transporters, especially efflux transporters may also play an important role in determining the overall distribution of intranasally administered compounds. The evaluation of *in vivo* disposition patterns is complicated by the activity of a variety of transporters, some identified and others unknown, on distribution, along with a lack of information regarding exact tissue concentrations and target concentrations that result in the saturation of a transporter. Additional factors, including off-target effects of the probe molecule or any inhibitors added to the experimental protocol also add to the complexity of investigating mechanisms controlling drug disposition. This is the first report to utilize hybrid imaging techniques to demonstrate the importance of epithelial transporters in the absorption and subsequent nose-to-brain distribution of a substrate drug compound. The conclusion that epithelial nucleoside transporters play an important role in directing a drug molecule into the systemic circulation or into the brain from the nasal cavity leads to many intriguing new possibilities to target drug compounds to the brain using the nose-to-brain pathways.

Conclusions

- PET imaging enables the visualization and quantification of drug distribution patterns and is a powerful tool to aid in the understanding of nose-to-brain uptake.
- ENT1, a facilitative transporter, is expressed in a number of tissues including the olfactory epithelium and the brain endothelium. FLT distribution patterns show that nucleoside transporters, including ENT1, play a key role in the distribution of transporter substrates between the nasal cavity and the brain via the olfactory bulb.
- Inhibition of ENT1 in the nasal mucosa decreases distribution of FLT into the olfactory bulb following nasal administration.
- Due to the action of the nucleoside transporters in other tissues, further disposition is affected by transporter location and concomitant substrate and inhibitor absorption and distribution patterns.

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Figure 1.
Percent dose administered per cc of tissue in the olfactory bulb (blue diamonds) and residual brain (red squares) after intranasal (IN) administration without pre-treatment with inhibitor. Mean values based on N = 3 rats with replicate data from 2 rats for 5 total measurements.

Figure 2.

Percent dose administered per cc tissue in the olfactory bulb after intranasal (IN) administration (plot A) and intravenous (IV) administration (plot B) without pre-treatment with inhibitor (blue diamonds), after intraperitoneal (IP) inhibitor (yellow triangles) and IN inhibitor (green circles). The “no inhibitor curve” in plot A (blue diamonds) is equivalent to the curve in Figure 1. The IP and IN inhibitor curves are based on N = 2 animals per condition. Note the nearly 20-fold higher Y-axis scale on plot A compared to plot B.

Figure 3.

Percent dose administered per cc tissue in the rest of brain after intranasal (IN) administration without pre-treatment with inhibitor (red squares), after intraperitoneal (IP) inhibitor (yellow triangles) and IN inhibitor (green circles) with comparison to IV administration without inhibitor (red triangles). The “no inhibitor curve” (red squares) is equivalent to the curve in Figure 1. The IP and IN inhibitor lines are based on $N = 2$ animals per condition. The IV/no inhibitor condition is based on 3 measurements in $N = 2$ animals. Excluding the early times (< 3 minutes), the average ratio between the IN FLT/IN NBMPR and IN FLT/no NBMPR was 2.3.

Figure 4.

Area-under-the-curve (expressed in fraction of dose × time (seconds)) from time= 0 to approximately 40 minutes (2400 seconds) (top), and partitioned from time = 0 to 20 minutes (middle) and from time = 20 to 40 minutes (bottom) for the time-activity curve (TAC) from the volume-of-interest (VOI) in the olfactory bulb under the specified conditions of route of administration (IN (top plot = dark blue) versus IV (top plot = light blue)) and inhibitor status (no inhibitor, IP inhibitor or IN inhibitor).

Figure 5.

Area-under-the-curve (expressed in fraction of dose $\times$ time (seconds)) from time= 0 to approximately 40 minutes (2400 seconds) (top), and partitioned from time = 0 to 20 minutes (middle) and from time = 20 to 40 minutes (bottom) for the time-activity curve from the VOI in the brain under the specified conditions of route of administration (IN (top plot = dark red) versus IV (top plot = light red)) and inhibitor status (no inhibitor, IP inhibitor or IN inhibitor). The difference in the Y-axis scale compared to Figure 4 is due to the significantly larger volume of the brain (~1.5 mL) relative to the olfactory bulb (<0.1 mL).

Figure 6.

Fraction of the administered FLT dose (%) in olfactory bulb (OB – top) and rest of the brain (bottom) as determined by PET imaging (red bars) and ex vivo (blue bars) methods by route of administration (IN or IV) and NBMPR route (IN or IP inhibitor). Time between last frame (35 – 40 minutes post-administration) and euthanasia was 11 – 12 minutes except for one of the IV FLT/IP inhibitor animals (44 minutes).