An *In Vitro* Model for the Comparative Evaluation of Bone Seeking Pharmaceuticals

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Summary

The absence of secured knowledge regarding the binding interaction between bone seeking pharmaceuticals and bone, rules out the possibility of modern rational drug design of novel bone seekers. Hence, the first step in the preclinical evaluation of any new bone seeker involves biodistribution and efficacy studies in laboratory animals. Here we report the development of a simple in vitro model for the comparative evaluation of new bone seekers. Additionally, we compared the binding characteristics found in our model with results obtained from animal experiments and evaluated the feasibility of our model to reduce or replace animal experiments in future. Correlations between our in vitro model and data from in vivo, ex vivo and cell culture studies support its applicability. On the basis of our findings we feel confident that this model could serve as a convenient alternative to animal experiments for the comparative evaluation of bone seekers. It could even be a basis for the development of a future pharmacopoeia-listed method allowing both the officinal quality control of bone seekers and a more respectful approach to animals.

Zusammenfassung: Ein *in vitro* Modell zur Evaluierung von knochenaffinen Arzneistoffen

Die genauen Mechanismen, welche Aufnahme und Verteilung von knochenaffinen Arzneimitteln bestimmen, sind bislang unbekannt. Diese Tatsache macht die Verwendung von Tierversuchen bereits in der Frühphase von Entwicklungsstudien notwendig und sogar das Europäische Arzneibuch sieht für die Qualitätskontrolle eine Untersuchung auf physiologische Verteilung in Ratten vor.

Der vorliegende Aufsatz beschäftigt sich nun mit einem Modell, welches in vitro die Bindung zwischen knochenaffiner Substanz und Knochenmatrix zu simulieren vermag. Dieses Modell wird kritisch auf seine Eignung untersucht, die Vorgänge im lebenden Organismus mit einfachen Mitteln nachvollziehen und simulieren zu können. Da die Datenlage eine derartige Eignung nahe legt, schlussfolgern die Autoren, dass die beschriebene Methode zukünftig Tierversuche in der Frühtestung neuer und in der vorschriftsmäßigen Reinheitstestung knochenaffiner Substanzen ersetzen könnte.

Keywords: bone scan, hydroxyapatite, bone seekers, polyphosphonate, model

1 Introduction

Since several primary tumours, such as those of breast, lung and prostate, are known to build metastases into osseous tissue, skeletal imaging and bone pain palliation represent major tasks in nuclear medicine. In clinical routine, a variety of radioactively labelled polyphosphonates (MDP, DPD , EDTMP, …) provide convenient and effective means to monitor disease progression or improve life quality of the patients, depending on the nature of the chosen radionuclide (Subramanian et al., 1975).

Either way, the minimisation of both the radiation burden to the patient and interfering signals in the scintigraphic image requires specific skeletal localisation of the radiopharmaceuticals (Billinghurst, 1982). Although bone seekers have been used for more than 30 years in nuclear medicine, the underlying mechanism involved in their accumulation in osseous tissue is still under discussion (Francis et al., 1980; Kanishi, 1993; Okamoto, 1995). Hypotheses regarding these mechanisms range from adsorption onto the mineral or organic phase of bone to incorporation into the mineralisation processes with disputed contribution of bone cells.

The absence of secured knowledge rules out the possibility of modern rational drug design, including the analysis of structure-activity relationships, in the development of novel bone seeking agents. Hence, the first step in the preclinical evaluation of any new bone seeker involves biodistribution and efficacy studies in laboratory animals. The demand of laboratory animals goes even further: the European pharmacopoeia specifies (European Pharmacopoeia, 2005) that as part of quality control each charge of the officinal polyphosphonate has to be tested regarding its biodistribution in 3 rats prior to release.

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Our aim was (1) to develop a simple *in vitro* model for the comparative evaluation of new bone seekers avoiding animals, (2) to compare the binding characteristics found in our model with results obtained from animal experiments and (3) to evaluate the feasibility of our model to reduce or replace animal experiments in future. The present manuscript is based on recently presented techniques and should help bringing up this new focus of reducing the demand for animal experiments. As the animal experiments for the evaluation of the usefulness of our model were only associated with a very low level of stress for the animals, we find they are justified, as they can save a large number of animals from future experiments.

Abbreviations

Co	human cortical bone powder
DPD	3,3-diphosphono-1,2-propanedicarboxylic acid
EDTMP	1,2-Ethanediylbis[nitrilobis-(methylene)]tetrakis-phosphonic acid
HA	hydroxyapatite
HBSS	Hank's balanced salt solution
MDP	methylene diphosphonate
n.c.a.	no carrier added
%ID/g	percent injected dose per gram of organ
PET	positron emission tomography
SUV	standardised uptake value
TAC	time activity curve

2 Experimental

2.1 Materials

Multibone® kits (containing 25 mg EDTMP in lyophilised form) were commercially obtained (Izotop, Budapest, Hungary). Hank's balanced salt solution (HBSS, H 8264) and hydroxyapatite (HA, 21223) were purchased from Sigma-Aldrich (Steinheim, Germany). Millex-SG 0.22 µm sterile filters were from Millipore (Bedford, MA, USA). Measurements of radioactivity were performed on a Cobra-II auto-gamma-counter (Canberra Packard, Canada). The thermostatic water bath 1083 was from GFL (Burgwedel, Germany), and the dose calibrator was a Curiementor 2 from PTW (Freiburg, Germany).

2.2 Preparation of human cortical bone powder (Co)

The preparation followed methods reported elsewhere (Kluger et al., 2003). Briefly, bone specimens were excised from donors, washed, freeze-dried and sterilised. Afterwards, these allografts were processed into a fine powder by milling. Measurements revealed a particle size in a comparable range to the commercially available HA.

2.3 Binding experiments

To a vial containing 3 mg of HA or Co 3 ml of HBSS were added and the vial was swayed at 37°C for 24 h. Radioactively labelled polyphosphonate (content of ligand: 0.3 µmol, based on commercially available radiolabelling kits) (Mitterhauser et al., 2004; Mitterhauser et al., 2005; Toegel et al., 2006a; Mitterhauser et al., 2007) or 25 MBq [^{18}F]-fluoride were added. Then the tube was returned to the water bath (120 min, 37°C) and vortexed every 15 min and before extraction.

2.4 Binding measurement

After 120 minutes of incubation an aliquot of 50 µl of this suspension was added to 2 ml of physiological saline. Out of this dilution, three aliquots of 50 µl were taken and placed in tubes for the gamma-counter. The rest of the dilution was filtered through a Millex-SG single use filter unit, and three aliquots of 50 µl were taken from the filtrate and placed in tubes. The radioactivity of the six tubes was measured in the gamma-counter, and the percentage of irreversibly bound radiolabelled PP was calculated as percent binding.

2.5 Filter experiments and filter correction

Filter experiments were performed to control for the amount of unspecific radioactivity retained during filtration. The procedure was similar to the *Binding Experiments* and *Binding Measurement*. The only modification was the omission of binding matrix and associated incubation periods. The blank values obtained from filter experiments were converted iteratively via the term:

$[FV_M + (100\text{-}V_M)]/100 * FV_M = FV_1$.

After 4 iterative arithmetic operations FV_4 was obtained and subtracted from V_M (=>V_{Fc}).

FV_M= Filter value, measured in filter experiment

$FV_{1\text{-}4}$= Filter values, calculated iteratively

V_M= Value, measured in binding experiment

V_{FC}= Value, filter corrected

2.6 microPET experiments

All experiments were approved by the Austrian law on animal experiments. All data were acquired on a microPET® Focus™ 220 tomograph (Siemens, Knoxville, TN) using six-week old female wild type Him:OF1 mice. The PET imaging field of view was 190 mm in diameter in the transverse by 76 mm in the axial direction. A butterfly catheter was placed in the tail vein. The mouse was positioned on the tempered animal bed (37°C) in the microPET scanner and kept under isoflurane anaesthesia (1.57%). After radiotracer administration (0.2 mL, 15 seconds) of 0.31-2.05 MBq [^{68}Ga]-EDTMP or 1.01-4.17 MBq [^{18}F]-fluoride, data acquisition was started. The dynamic image data (energy window: 250–750 keV; timing window: 6 ns) were sorted into 3-dimensional sinograms (frames: 7x1 min, 4x2 min, 3x5 min, 3x10 min, 8x15 min). All sinograms were Fourier transformed into 2D sinograms prior to reconstruction. Dynamic images were reconstructed using 2D filtered back projection with a ramp filter cut-off at the Nyquist frequency. Transmission scans using a Co-57 point source were performed for 10 minutes. Emission data were corrected for detector efficiency, random coincidences, dead time, isotope decay and attenuation. The PET image volume (128x128x95) was reconstructed with a zoom of 6 and had a voxel size of 0.32x0.32x0.8mm^3.

2.7 Biodistribution experiments

After microPET scans all animals were killed by cervical dislocation, organs (femurs, tail, liver, kidneys) were dissected within a few minutes, weighed and subjected to gamma-counting. The percentage of injected dose per gram tissue

(%ID/g) was calculated using two calibration curves (high and low activity) with known activities and decay corrected for the injection time.

2.8 Statistics

Statistical analyses were performed using the Microsoft Excel integrated analysis tool. Hypothesis tests among two data sets were made by comparison of two means from independent (unpaired) samples (t-test). A value of $p<0.05$ was considered significant. Descriptive statistical analyses were performed using mean values and standard deviations.

3 Results

3.1 Binding experiments

Results of the binding experiments on HA are presented in Table 1. Values range from 4.29% ± 2.74 ([^{68}Ga]-EDTMP) to 71.66% ± 9.32 ([^{99m}Tc]-MDP). It is evident that uptake depends on the structure of the polyphosphonate ligand, the radionuclide used for radiolabelling and the nature and amount of carrier. Table 2 shows the uptake of several no carrier added preparations (n.c.a.) on human corticalis. Here, values range from 5.44% ± 1.99 ([^{68}Ga]-EDTMP) to 65.29% ± 4.73 ([^{18}F]-fluoride). Again, uptake strongly depended on the structure of the polyphosphonate ligand and the radionuclide.

3.2 microPET experiments

The time activity curves (TACs) of [^{68}Ga]-EDTMP and [^{18}F]-fluoride in humerus and femur are presented in Figure 1. Highest uptake was found in femurs of the mice administered with [^{18}F]-fluoride (5.07 at 172 minutes). Values obtained from [^{18}F]-fluoride were significantly higher than those obtained with [^{68}Ga]-EDTMP ($p<0.01$).

3.3 Biodistribution experiments

Results are presented in Table 3. It is evident that accumulation of [^{18}F]-fluoride in the femur was significantly higher ($p<0.01$) as compared to that of [^{68}Ga]-EDTMP (both SUV (standardised uptake value) and %ID/g), whereas no significant difference for all the other organs was observed.

4 Discussion

Although the exact mechanisms of bone uptake are discussed controversially, the involvement of the inorganic bone compartment in this process seems to be undisputed. In autoradiographic analyses of foetal calvarias, Kanishi (1993) found high uptake in the area of the osteoblast-like cells, whereas no uptake was found in osteoblast-like cells themselves *in vitro*. Francis et al. described the involvement of inorganic structures in the drastically increased uptake in osteoblastic bone pathologies (Francis and Fogelman, 1987; Francis et al., 1980). In contrast, it is known that areas of osteoclastic activity show reduced uptake. Since uptake of bone seekers is presumably restricted to the mineral phase of bone, we developed an *in vitro* model for the evaluation of this bone seeker – matrix interaction (Mitterhauser et al., 2004; Mitterhauser et al., 2005). Based on our studies and an in-depth survey of the results, we developed the hypothesis that our model may either reduce or even replace animal experiments in the field of radioactive bone seekers. Up to the present, these animal experiments are demanded for quality control procedures (European Pharmacopoeia, 2005) and are supposed to be mandatory during tracer development. The present manuscript was designed to suggest our model as an alternative to animal experiments for the prediction of *in vivo* binding characteristics. In order to substantiate this hypothesis, the following correlations are presented:

(1) The model reflects hydroxyapatite binding characteristics in mineralising osteoblast cell culture. In an experimental set up, where osteoblasts from mouse calvariae were cultured under forced mineralising conditions, we quantified binding of several bone seekers (Toegel et al., 2006b). We found a good correlation between the binding values determined in our *in vitro* model and those observed in the cultures. Since we could verify that binding took place solely on hydroxyapatite and not on the osteoblasts themselves, this comparison revealed that our model could reflect and replace studies on these primary animal cells.

(2) The model reflects binding characteristics on human ex vivo bone allografts. Comparing Table 1 and Table 2, it is evident that uptake rankings of all n.c.a. formulations based on EDTMP are in the same order Ga < Tc < Re < In < Y < Sm. In addition, [^{99m}Tc]-MDP yielded highest binding of the ligand based preparations on both human and synthetic binding matrices. Furthermore, carrier addition resulted in increased binding values of [^{99m}Tc]-EDTMP, [^{68}Ga]-EDTMP and [^{111}In]-EDTMP on both matrices, whereas in the case of [^{188}Re]-EDTMP carrier addition resulted in reduced binding on

Tab. 1: Shows the % binding of the PP-based tracers (0.3 µmol) and n.c.a. [^{18}F]-fluoride on 3 mg HA after 120 minutes.
Data were collected using the filtration method described in the *Methods* section. Each value represents the filter corrected arithmetic mean value of at least 5 experiments, each performed in triplicate.

Tracer	Mean±SD
[^{99m}Tc]-MDP	71.7±9.3
[^{99m}Tc]-DPD	29.9±5.0
[^{99m}Tc]-EDTMP	8.9±2.3
[^{99m}Tc]-/Re-EDTMP (11 µl Re-carrier added)	13.1±2.9
[^{99m}Tc]-/Re-EDTMP (15 µl Re-carrier added)	19.0±0.7
[^{99m}Tc]-/Re-EDTMP (80 µl Re-carrier added)	31.2±2.5
[^{99m}Tc]-/Re-EDTMP (150 µl Re-carrier added)	43.1±1.6
[^{99m}Tc]-/In-EDTMP	13.6±3.0
[^{99m}Tc]-/Y-EDTMP	8.4±3.6
[^{188}Re]-EDTMP	23.0±3.5
[^{188}Re]-/Re-EDTMP	9.4±0.7
[^{111}In]-EDTMP	25.3±3.2
[^{111}In]-/Re-EDTMP	24.9±2.5
[^{111}In]-/In-EDTMP	28.8±2.6
[^{111}In]-/Y-EDTMP	17.9±2.7
[^{90}Y]-EDTMP	28.2±2.1
[^{90}Y]-/Re-EDTMP	30.6±4.1
[^{90}Y]-/In-EDTMP	28.0±2.1
[^{90}Y]-/Y-EDTMP	21.9±3.4
[^{68}Ga]-EDTMP	4.3±2.7
[^{68}Ga]-/Re-EDTMP	9.5±2.1
[^{68}Ga]-/In-EDTMP	6.9±1.7
[^{68}Ga]-/Y-EDTMP	6.7±0.9
[^{68}Ga]-/Ga-EDTMP	5.6±2.4
[^{153}Sm]-EDTMP	52.3±1.0
[^{18}F]-fluoride	67.8±1.4

both matrices (Mitterhauser et al., 2005; Toegel et al., 2006a; Toegel et al., 2007). These facts strengthen the use of hydroxyapatite as a surrogate for human bone allografts.

(3) The model reflects bone binding characteristics in rodents ex vivo. Ex vivo animal studies are currently specified for the evaluation of physiological distribution and purity of officinal bone tracers such as [^{99m}Tc]-MDP. In the present manuscript, we provide *ex vivo* data on such a biodistribution experiment performed with [^{68}Ga]-EDTMP and [^{18}F]-fluoride in mice. Table 3 indicates that [^{18}F]-fluoride accumulated to a significantly higher extent ($p<0.01$) in osseous tissue (femur) in comparison to [^{68}Ga]-EDTMP (2.9-fold increase of %ID/g and 2.8-fold increase in SUV). Of note, the results obtained from our *in vitro* model also show the binding superiority of [^{18}F]-fluoride over [^{68}Ga]-EDTMP (Tab. 1). The findings indicate that a comparative evaluation of bone seekers was successfully performed using our *in vitro* model and that the results from those *ex vivo* animal studies were congruently reproduced. In a different *ex vivo* study, Láznícek et al. (1994) presented a comparison of biological characteristics between EDTMP complexes radiolabelled with [^{99}mTc], [^{111}In] and [^{153}Sm] in rats showing the same order of uptake as we did using our HA-based model (Tab. 1): [^{99}mTc]-EDTMP < [^{111}In]-EDTMP < [^{153}Sm]-EDTMP.

(4) The model reflects bone binding characteristics in rodents in vivo. For a new PET-tracer, prior to its application in humans, the visualisation of major distribution pathways in laboratory animals can be a helpful tool. Nowadays, microPET – as the most advanced imaging technology – provides a means to conduct animal experiments in an accurate and efficient manner. In particular, its ability to perform longitudinal studies on the same animal has certain advantages over *ex vivo* studies. However, in our

Tab. 2: Shows the % binding of the PP-based tracers (0.3 µmol) and n.c.a. [^{18}F]-fluoride on 3 mg Co after 120 minutes.
Co was prepared from human bone allografts by milling. Data were collected using the filtration method described in the *Methods* section. Each value represents the filter corrected arithmetic mean value of at least 5 experiments, each performed in triplicate.

Tracer	Mean±SD
[^{99m}Tc]-MDP	31.7±4.3
[^{99m}Tc]-EDTMP	5.7±1.3
[^{188}Re]-EDTMP	7.7±2.3
[^{111}In]-EDTMP	11.4±11.8
[^{90}Y]-EDTMP	24.8±2.4
[^{68}Ga]-EDTMP	5.4±1.9
[^{153}Sm]-EDTMP	27.8±2.8
[^{18}F]-fluoride	65.3±4.7

Tab. 3: Shows the percent injected dose per gram organ (%ID/g; mean values ± SD; n ≥ 3) or SUV (mean values ± SD; n ≥ 3) in various organs after injection of [^{18}F]-fluoride or [^{68}Ga]-EDTMP.
Organs were dissected from sacrificed mice, weighed and subjected to gamma-counting. %ID/g was calculated using two calibration curves (high and low activity) with known activities and decay corrected for the injection time.

Organ	[^{18}F]-fluoride		[^{68}Ga]-EDTMP	
	%ID/g± SD	SUV*± SD	%ID/g± SD	SUV*± SD
Femur	13.3±2.8	3.1±0.6	4.6±1.1	1.1±0.2
Tail	4.7±1.8	1.1±0.4	4.7±4.8	1.2±1.2
Liver	< 0.05	< 0.01	< 0.01	< 0.01
Kidneys	< 0.01	< 0.01	< 0.01	< 0.01

* SUV (Standardised Uptake Values) calculated as: $(Bq/g_{(Organ)})/Bq_{(administered)}*g_{(weight\ of\ mouse)}$.

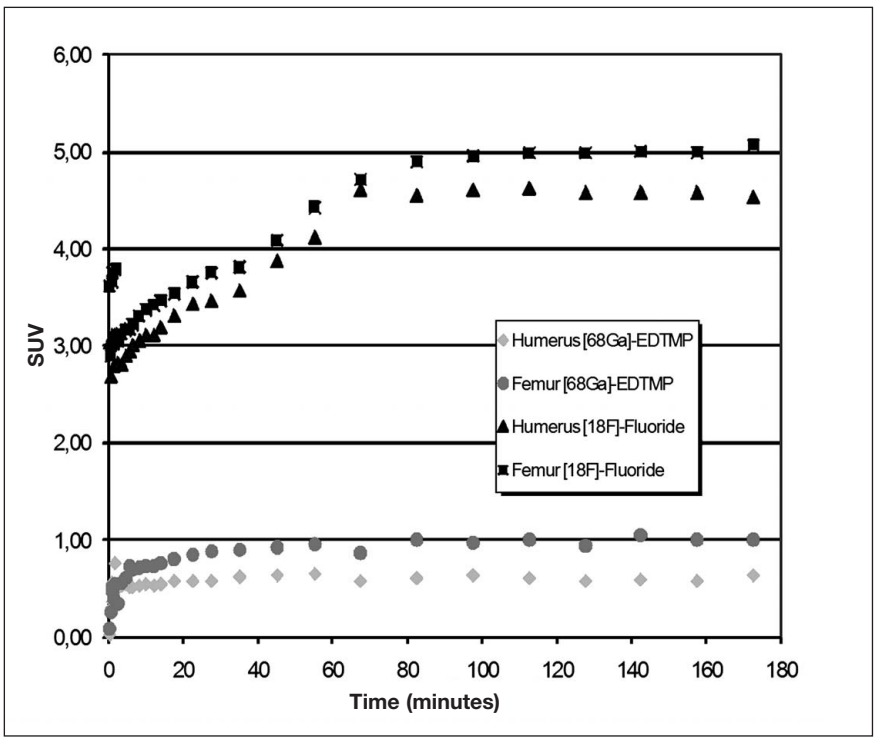

Fig. 1: Shows the time activity curves of [^{18}F]-fluoride and [^{68}Ga]-EDTMP in humerus and femur in mice.
On the reconstructed microPET images regions of interest (ROI) were drawn manually using the Image Quantification and Kinetic Modeling Software PMOD 2.7. Tracer uptake in these ROIs was quantified as standardised uptake values (SUV) using the formula: $(Bq/g_{(Organ)})/Bq_{(administered)}*g_{(weight\ of\ mouse)}$.

view, the radiation burden and the current animal welfare laws – which regulate the killing of laboratory animals after experiments – beg a suitable alternative. Figure 1 shows the uptake values of [^{68}Ga]-EDTMP and [^{18}F]-fluoride in bone derived from reconstructed microPET. Again, the order of uptake is in close agreement with our *in vitro* model.

(5) The model reflects bone binding characteristics in man. In a previous study on patients, we evaluated [^{99m}Tc]-/Re-EDTMP and compared the findings with [^{99m}Tc]-DPD and n.c.a. [^{99m}Tc]-EDTMP (Füger et al., 2004; Mitterhauser et al., 2001). We found uptake in the order: n.c.a. [^{99m}Tc]-EDTMP < [^{99m}Tc]-/Re-EDTMP (11 µl) < [^{99m}Tc]-DPD. These data were reproduced in our model (Tab. 1).

5 Conclusion

The present report describes the use of an *in vitro* model for bone seekers based on simple binding to hydroxyapatite and its application for evaluating bone binding characteristics. On the basis of our findings we feel confident that this model could serve as a convenient alternative to animal experiments for the comparative evaluation of bone seekers. It could even be a basis for the development of a future pharmacopoeia-listed method allowing both the officinal quality control of bone seekers and a more respectful approach to animals.

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