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Comparison between two labeled agents in mice using a coinjection-ratio approach in contrast to a conventional group approach

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Abstract

Introduction—The differences between two agents often need to be accurately defined in vivo. Conventionally they are injected respectively into two groups of subjects. However, if the two agents do not interact with each other in vivo, a coinjection would serve the same purpose. We believe some individual differences in biodistribution may be circumvented through this approach by calculating organ level ratios.

Methods—A model system of MORF/cMORF pretargeting (MORF/cMORF is a complementary pair of DNA analogues) was employed in connection with an on-going tumor therapeutic project. Human LS174T cells were implanted into the flank of severely immuno-compromised NOD-*scid* *IL2rg^{null}* mice. The tumor was confirmed to express TAG-72 antigens. At 16 days post tumor inoculation, mice received IV 60 µg of MORF-conjugated CC49 (an antiTAG-72 antibody), followed 2 days later by a low-mass-dose IV coinjection containing 2.5 µg of ⁹⁰Y-cMORF and 2.5 µg of ^{99m}Tc-cMORF. At 3 h post radioactivity injection, the distribution of ^{99m}Tc was imaged on a SPECT/CT camera and then organs were excised and counted for ⁹⁰Y and ^{99m}Tc. Because the two labeled cMORFs do not react or interact with each other in vivo, the two groups of ⁹⁰Y and ^{99m}Tc data enabled a conventional group comparison. In a new effort, ⁹⁰Y/^{99m}Tc ratios were calculated. Student's t-test and retrospective power analysis were performed for both approaches. In the new approach, the ratios were set at 1 as the null hypothesis.

Results—The student's t-test in the conventional group approach indicated that the two labeled cMORFs distributed similarly, but significant differences were observed in salivary gland and large intestines. The coinjection-ratio approach certainly did not subvert the results of the conventional approach but revealed subtler differences. The P values were reduced, the powers

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were increased in most organs, and more significant differences were observed. The increased sensitivity was due to the reduced CV%_s of the $^{90}\text{Y}/^{99\text{m}}\text{Tc}$ ratios ($\text{SD}/\text{Average} \times 100\%$). Therefore, some individual differences were circumvented and notably the ratio approach differentiated individual differences into ratio-correctable and ratio-uncorrectable.

Conclusions—Although the conventional approach is reliable, the coinjection-ratio approach using level ratios is more sensitive and therefore is recommended whenever possible. In addition, it differentiates individual differences into “coinjection correctable” and “coinjection uncorrectable”.

Keywords

Statistical analysis; in vivo evaluation; biodistribution; translational study; tumor pretargeting

INTRODUCTION

In many pharmaceutical investigations, the difference between two agents needs to be accurately defined. Two groups of subjects are often needed under each condition or time point and the student's t-test is the most widely used statistical analysis. The number of groups and the number of animals in each group may vary depending on the variance of the data or the aim of the studies. For example, the methodology to define the difference between two samples (two agents or two conditions) for radiopharmaceutical development has been well described [1–2]. The use of an adequate number of animals in each group is to offset the individual variations.

In some radiopharmaceutical studies, the measure is %ID/g or %ID and the biodistribution data are compared. If one agent does not interact with the other in vivo, a coinjection may be performed, depending on whether the signals from the two agents can be separated. In addition to saving animals, the coinjection allows for calculating the organ level ratios. We believe the level ratios as new measures should circumvent some of the individual differences. For example, in tumor studies, the inconsistency in tumor size and location add to the intrinsic individual differences of animals, but a tumor that highly accumulates one agent should also highly take up the other if the two function identically. In this case, the ratios should remain constant among animals, i.e. the variance of the ratios should be minimal. In other words, we hypothesize this coinjection-ratio approach is more sensitive than the conventional group approach for comparing two agents.

In connection with an on-going tumor therapeutic project that employed a pretargeting strategy using a pair of mutually complementary phosphorodiamidate morpholino oligomers (MORF/cMORF), we compared a $^{99\text{m}}\text{Tc}$ -labeled cMORF ($^{99\text{m}}\text{Tc}$ -cMORF) as the diagnostic agent and a ^{90}Y -labeled cMORF (^{90}Y -cMORF) as the therapeutic agent. We previously found the cMORFs labeled with different nuclides excrete through kidneys and share similar pharmacokinetics [3]. However, it was occasionally reported that a label change does not assure an unchanged pharmacokinetics [4–6]. To accurately define the differences between the $^{99\text{m}}\text{Tc}$ -cMORF and the ^{90}Y -cMORF in tumor-targeting, we have designed a coinjection study using tumor-bearing mice that are pretargeted by a MORF-modified antibody. This design may serve as a model to validate the above hypothesis that the ratio approach is more sensitive.

MATERIALS AND METHODS

The $^{90}\text{YCl}_3$ and the ^{99}Mo - $^{99\text{m}}\text{Tc}$ generator were from Perkin Elmer Life Science Inc (Boston, MA). The MORF (5'-TCTTCTACTTCACAATA) and cMORF (5'-TAGTTGTGAAGTAGAAGA) were obtained from Gene-Tools (Philomath, OR) with

amine-derivatization at the 3'-equivalent terminus. The NH₂-cMORF was conjugated with p-SCN-Bn-DOTA (Macrocyclics, Dallas, TX) for ⁹⁰Y labeling [3] and with NHS-MAG₃ for ^{99m}Tc labeling [7]. The murine anti-TAG-72 antibody CC49 was prepared by Strategic Biosolutions (Ramona, CA) from the CC49 hybridoma (a gift from Dr. Jeff Schlom, Laboratory of Tumor Immunology and Biology, Center for Cancer Research, NCI, NIH, Bethesda, MD). The antibody was conjugated with MORF using a commercial Hydralink approach as previously described [8–9]. All other chemicals were reagent grade and used without purification.

Tumor model

All animal use was approved by the Animal Care and Use Committee of the University of Massachusetts Medical School. Previously, NIH Swiss nude mice were the routine strain for the development of the LS174T tumor model [10]. In the current study, NOD.Cg-*Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ* mice (abbreviated as NOD-*scid* *IL2rg^{null}* or NSG) were used [11], considering they readily accept engrafted human tumor tissues without changing the pathological texture and therefore are potentially useful for advancing imaging technology to primary tumors in future. The mice were obtained from the colony of Dr. Leonard Shultz at The Jackson Laboratory (Bar Harbor, ME). The LS174T tumor was inoculated in the flank and the animals were entered into the experiments 16 days later. The presence of the TAG-72 antigens in this model was histochemically confirmed as previously described [12], but using the CC49 antibody instead of the previous B72.3 antibody.

Biodistributions of the labeled cMORFs in pretargeted mice

Theoretically, comparison between the two labeled cMORFs requires multiple time points in both normal mice and pretargeted mice with tumors. However, we have determined previously that the labeled cMORFs distribute similarly [3]. Furthermore, the data at one time point should be sufficient for validating the improved sensitivity of the ratio approach. Finally, the concern in the related project is whether the final biodistribution of the diagnostic agent in pretargeted animals represents that of the therapeutic agent. Hence, only one time point was chosen at 3 h when the unbound cMORFs had cleared almost completely.

Following a previously optimized pretargeting schedule [13], four NSG mice bearing LS174T tumors in the left flank received intravenously 60 µg of MORF-CC49 (MORFs per CC49 = 0.68, 0.255 nmol MORF), two days prior to injecting a solution that contained 2.5 µg (0.40 nmol) of ⁹⁰Y-cMORF (0.05 mCi) and 2.5 µg (0.40 nmol) of ^{99m}Tc-cMORF (0.8 mCi). The molar dose ratio of cMORF/MORF (1.56) was only half of that (cMORF/MORF = 3.10) previously determined without a dose-effect [9, 14]. Thus, the total cMORF mass dose was sufficiently low for freely binding to the MORF without competition and the two labeled cMORFs were functioning independently. The animals were imaged and euthanized at 3 h post-injection of the radioactivity prior to dissection. A small animal multiple pinhole SPECT/CT camera (Bioscan Inc., Washington D.C., USA) was used to perform the mouse imaging under isoflurane, by acquisition of the ^{99m}Tc signals at 60s/projection (total 24 projections). Samples of blood and tissues of interest were collected at necropsy, weighed, and counted in a NaI(Tl) auto-gamma well counter along with the injectate standards in two respective channels (100–200 Kev for ^{99m}Tc and 200–1200 Kev for ⁹⁰Y). For ⁹⁰Y counting, the tissues were digested following a previously reported procedure [3] and were measured immediately and then 3 days later when the ^{99m}Tc activity decayed almost completely.

The net counts were calculated. For the ⁹⁰Y counts, the data after the ^{99m}Tc had decayed were used. For the ^{99m}Tc counting, the simpler way was to use the day-1 counts minus that calculated back from day 4-counts using the ⁹⁰Y half-life ($t_{1/2} = 64$ h) and the formula

$A_{0\text{ net}} = A_0 - A_{\text{day4}} \times \text{Exp}(0.6932 \cdot T/64\text{h})$. A faster way to get the net $^{99\text{m}}\text{Tc}$ counts before its decay was to count the samples twice in about 3 h, using the formula of $A_{0, \text{net}} = (A_0 - A)/[1 - \text{Exp}(-0.6932 \cdot T/6.007 \text{ h})]$, as the ^{90}Y contribution to the $^{99\text{m}}\text{Tc}$ window essentially did not change in this short period of time. Both approaches were tested and no difference was found.

Statistical methods

Because the two labeled cMORFs do not interact with each other and the total mass dose was sufficiently low, the data from the coinjection were equatable to those from a conventional group design using two groups of mice. Therefore, an unpaired comparison by a student's t-test was performed conventionally between the $^{99\text{m}}\text{Tc}$ -cMORF and the ^{90}Y -cMORF. Alternatively, in a new effort, the $^{90}\text{Y}/^{99\text{m}}\text{Tc}$ ratios were calculated. They intuitively represent the differences and should circumvent some individual differences. Student's t-test was applied to this approach along with the conventional approach, but setting the ratios at 1 as the null hypotheses and assuming the CV%s were the same. In both analyses, a two-tailed test was performed because prior to the study it was known that most organ levels of the $^{99\text{m}}\text{Tc}$ -cMORF and the ^{90}Y -cMORF were similar [3]. The sample size of the choice was a prevailing number of $N=4$ used in the field. Perspective power analysis for size selection was not performed because the aim of the current investigation was to demonstrate a more sensitive coinjection-ratio approach rather than to define the difference of the two agents in one organ using the smallest adequate sample size. Retrospective power analysis was conducted at the end of the experiment to demonstrate the power by each method in each organ for differentiating the $^{99\text{m}}\text{Tc}$ -cMORF and the ^{90}Y -cMORF.

RESULTS

Tumor growth and TAG-72 expression

The LS174T tumors grew slightly slower in the flank of NSG mice than in the thigh of the NIH Swiss nude mice [15], taking about 18 days as compared to 11–12 days to reach the same average size of 0.5 g. The distribution of the TAG-72 antigens throughout the LS174T tumor in the NSG mice was essentially uniform, similar to that observed in the NIH Swiss nude mice [12].

Conventional group comparison

The averages, SDs, and the CV%s (percent Co-efficient Variation, i.e., $\text{SD}/\text{Average} \cdot 100\%$) of the $^{99\text{m}}\text{Tc}$ and ^{90}Y organ levels in the pretargeted mice at 3 h are listed in Table 1. The P values from an unpaired and two-tailed student's t-test are listed in Table 2. This conventional comparison indicates that the biodistribution difference between the $^{99\text{m}}\text{Tc}$ -cMORF and ^{90}Y -cMORF is insignificant in most organs except for the salivary gland and large intestines under the criteria of $P < 0.05$. The slightly higher level of $^{99\text{m}}\text{Tc}$ -cMORF in salivary gland is probably due to the presence of trace amount of pertechnetate. The 4-fold higher accumulation in the large intestines has been known to be due to the ~2% hepatobiliary excretion of the $^{99\text{m}}\text{Tc}$ -cMORF previously observed [3, 16].

Coinjection-ratio comparison

Unlike the conventional approach, coinjection of the two labeled cMORFs does not require grouping and allows for calculating the $^{90}\text{Y}/^{99\text{m}}\text{Tc}$ ratios in organs and tumor. The ratios, their averages, SDs, and CV%s are listed in Table 1 and the relative effect sizes [defined as $(\text{Ave}_1 - \text{Ave}_2) \cdot 100\% / \text{Ave}_1$], the two-tailed P values, and the powers from a retrospective power analysis are listed in Table 2 along with those for the conventional group approach.

Certainly, the coinjection-ratio approach does not subvert the conclusions of the conventional approach as evident by the comparable or essentially the same relative effect sizes. However, this approach reveals subtler changes. The P values for all organs except for the spleen and tumor are considerably reduced as shown in Table 2. As a result, Student's t-test now exhibits significant differences in the heart, muscle, stomach, and small intestines ($P < 0.05$) in addition to the salivary and large intestines exhibiting significant difference in the conventional approach. In parallel, the powers ($100\% - \beta$, at a confidence level $\alpha = 5\%$) are also improved. Fig 1 recalls the basic concept of power analysis. Apparently, the further apart the two hypotheses are (larger $\mu_1 - \mu_2$), the less chance to commit a type II error (smaller β) and the more sensitive the test is (larger power, $1 - \beta$). In other words, a larger $\mu_1 - \mu_2$ defines an improved sensitivity. Because $\mu_1 - \mu_2$ correlates positively with (effect size/SD) or (relative effect size/CV%) and the relative effect size are the same, the improved sensitivity for the coinjection-ratio approach is due to the improved CV%s.

The ^{99m}Tc -cMORF surrogate imaging

As a direct application of the above results, the ^{99m}Tc image of a pretargeted mouse can be used as a surrogate of that of the non-imageable ^{90}Y , if bearing in mind those clearly defined differences. As shown in Fig 2, the hot spots in the intestines arise from the ~2% hepatobiliary excretion of the ^{99m}Tc -cMORF and are not representing the ^{90}Y levels. The ^{99m}Tc signals from lung and spleen may also deviate from the ^{90}Y accumulations to some extent, but they are not sufficiently intense for visualization and the uncertainty is limited to a slight background increase.

DISCUSSION

A group design of injecting the two agents separately is commonly used for a comparison between two agents, but if possible, this investigation recommends a coinjection (one-sample) approach because, in addition to saving animals, coinjection allows calculation of level ratios to circumvent some individual differences and to make a more sensitive comparison. There are three elements in the coinjection-ratio approach, i.e. coinjection, ratio calculation, and statistical analysis. The coinjection should be of common interest, because whenever the energy peaks and/or the half-lives of two nuclides are sufficiently different, with an auto-gamma counter, the counts from multiple channel countings at different decay times can be mathematically resolved to provide the net signals from the two agents. Pairs of therapeutic and diagnostic agents may include the nuclide pairs of (^{18}F , ^{67}Ga , ^{99m}Tc , ^{111}In , or ^{123}I , ^{125}I)/(^{90}Y , ^{188}Re , or ^{131}I). Furthermore, pairs of diagnostic nuclides are also applicable, for examples (^{18}F or ^{67}Ga)/(^{99m}Tc or ^{111}In), that expand the application of coinjection to in vivo PET/SPECT imaging. Finally, the type of signals may extend beyond radioactivity, such as optical and other signals, if measurable by other means.

The ratio calculation enables correction for some individual differences. The ratios reflect intuitively the differences between two agents. Furthermore, based on whether the individual differences can be corrected, ratio calculation differentiates them into “coinjection-correctable” and “coinjection-uncorrectable”. This differentiation further provides insights into the nature of the correctability. It seems that an individual difference is correctable if it varies in parallel between the two agents. Take the tumors examples, the tumor accumulations are different due to the variance in size, texture, receptor distribution, and blood supply. However, the ratios are almost the same ($^{99m}\text{Tc}/^{90}\text{Y} = 1.00 \pm 0.01$). The same tumor ratio can be explained by the same blood ratio ($^{99m}\text{Tc}/^{90}\text{Y} = 1.01 \pm 0.02$) based on a theory previously described [17]. Another example is for the muscle levels. The values for mouse number one deviate seriously from the mean, leading the CV%s to as high as 62% and 63%. However, there is almost no difference in ratios ($^{99m}\text{Tc}/^{90}\text{Y} = 0.96 \pm 0.02$, CV% = 2%). The deviation was likely due to urine contamination during dissection. The $^{99m}\text{Tc}/^{90}\text{Y}$

ratio in the urine is equal to that in muscle and therefore the contamination is not changing the ratios.

Some individual differences must be non-correctable because the CV%*s* of the level ratios remain large. Theoretically those individual differences in the *in vivo* biological processes such as metabolic process are not correctable. For example, in the intestines, the total amounts of ^{99m}Tc getting into the intestinal tract were almost equal (calculated to be 3.83, 3.63, 3.63, and 3.66 %ID for small plus large intestines) probably by virtue of stable liver functions. However, the total amounts for the ⁹⁰Y were varying and essentially proportional to the blood level at an intestine/blood ratio of 0.55 ± 0.01 (after excluding the mouse number that exhibits a 0.83). Food contamination was suspected for this mouse, judged by the high stomach level. As the total levels of ⁹⁰Y and ^{99m}Tc do not vary in parallel, their ratios (calculated as 0.38, 0.26, and 0.30) are not a constant (mouse number three excluded). Furthermore, probably due to the individual differences in postprandial time at euthanization, the separate ^{99m}Tc levels in small or large intestines vary although the totals do not.

There may be instances that the differences can be largely but not completely eliminated, such as in the stomach. Agent difference exists as the ⁹⁰Y/^{99m}Tc ratios (~ 0.65) deviate significantly from 1. The ingestion of urine-contaminated food for mouse number three increased the stomach levels, but the contaminations by both nuclides are to the same extent (⁹⁰Y/^{99m}Tc = 1 in urine) instead of at a ratio equal to the intrinsic ~ 0.65 . Thus, the influence cannot be completely eliminated, although the variance of the ratios is much reduced (CV%*s* of 84% for ^{99m}Tc and 99% for ⁹⁰Y vs 18% for the ⁹⁰Y/^{99m}Tc).

In this investigation, the same statistical analysis was used with the same animals. These conditions allowed us to focus solely on the merit of ratio calculation in the improvement of the CV%*s* with no interference from other factors, such as inconsistent experimental quality between two groups of animals (the extent in controlling the experimental conditions, tumor size, etc.). Inconsistent quality is often a factor in the conventional design using two groups of subjects.

CONCLUSION

Coinjection of two agents plus the calculation of level ratios makes a more sensitive comparison than conventionally injecting them to two separate groups. In addition, the coinjection-ratio approach differentiates individual differences into “coinjection correctable” and “coinjection uncorrectable”. The data from this investigation suggest the use of the coinjection-ratio approach whenever possible for more accurate comparison.

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Fig 1.

A schematic recalling the principle of power analysis, showing that the more separated the null and alternative hypotheses are, the smaller the β and the larger the power.

Fig 2.

A SPECT/CT infused image of one mouse. Only the ^{99m}Tc signal is visible because the ^{90}Y as a pure β emitter does not contribute. The white arrows point to the LS174T tumor and the yellow arrows identify the intestinal radioactivity. Activity injected: 0.8 mCi of ^{99m}Tc and 0.05 mCi of ^{90}Y ; Anesthesia used: isoflurane; Acquisition time: 60s/per projection, 24 projections.

Table 1

Biodistributions of ^{90}Y -cMORF and $^{99\text{m}}\text{Tc}$ -cMORF in the pretargeted NSG mice at 3 h along with level ratios. Average $\pm$ SD; N= 4.

Organ	Tc-99m							Y-90							Y-90/Tc-99m							
	%ID/g	#1	#2	#3	#4	Ave	SD	CV%	#1	#2	#3	#4	Ave	SD	CV%	#1	#2	#3	#4	Ave	SD	CV%
Liver		1.02	0.84	0.86	0.64	0.84	0.16	19	1.06	0.90	0.85	0.73	0.88	0.14	15	1.03	1.07	0.99	1.15	1.06	0.07	6
Heart		0.52	0.37	0.30	0.34	0.38	0.10	26	0.57	0.40	0.32	0.35	0.41	0.11	27	1.10	1.08	1.08	1.05	1.08	0.02	2
Kidney		6.78	5.50	4.29	3.52	5.03	1.43	28	6.62	5.33	4.40	3.50	4.96	1.33	27	0.98	0.97	1.03	0.99	0.99	0.03	3
Lung		1.34	0.93	0.87	0.97	1.03	0.21	21	1.49	1.41	1.00	1.19	1.27	0.22	17	1.11	1.51	1.16	1.23	1.25	0.18	14
Spleen		0.57	0.43	0.36	0.40	0.44	0.09	21	0.60	0.59	0.67	0.42	0.57	0.11	19	1.05	1.36	1.86	1.04	1.33	0.38	29
Muscle		0.36	0.17	0.11	0.12	0.19	0.12	62	0.35	0.16	0.10	0.12	0.18	0.11	63	0.97	0.96	0.94	0.98	0.96	0.02	2
Tumor*		5.04	3.54	4.49	4.72	4.45	0.65	15	5.11	3.51	4.46	4.67	4.44	0.67	15	1.01	0.99	0.99	0.99	1.00	0.01	1
Pancreas		0.41	0.30	0.21	0.20	0.28	0.10	34	0.38	0.29	0.18	0.21	0.27	0.09	34	0.94	0.97	0.87	1.01	0.95	0.06	7
Sal. gland		0.36	0.41	0.34	0.39	0.37	0.03	8	0.30	0.30	0.29	0.28	0.29	0.01	4	0.82	0.73	0.86	0.72	0.78	0.07	9
Blood		2.49	1.74	1.85	2.00	2.02	0.33	16	2.55	1.75	1.81	2.03	2.04	0.36	18	1.02	1.01	0.98	1.02	1.01	0.02	2
Stomach		0.28	0.18	0.72	0.12	0.33	0.27	84	0.23	0.11	0.66	0.08	0.27	0.27	99	0.82	0.63	0.91	0.65	0.75	0.14	18
Sm. Int.		1.13	0.83	0.72	0.41	0.77	0.30	39	0.83	0.53	0.61	0.39	0.59	0.19	32	0.74	0.64	0.85	0.95	0.79	0.13	17
L.g. Int.		2.70	2.80	2.91	3.25	2.92	0.24	8	0.61	0.43	0.89	0.71	0.66	0.19	29	0.23	0.15	0.30	0.22	0.22	0.06	28

* The tumor sizes for mouse #1–4 are respectively 0.26, 0.55, 0.81, and 0.30 g.

Table 2

Statistical analysis results for both the conventional group approach and the conjunction ratio approach.

Organ	Conventional group approach			Conjunction ratio approach		
	(Ave _{TC} -Ave _Y)/Ave _{TC} (%)	P*	Power* (%)	(1-Ave _{Y/TC})/1 (%)	P*	Power* (%)
Liver	5	0.68	6	6	0.19	22
Heart	8	0.69	6	8	0.00	>95%
Kidney	1	0.95	5	1	0.55	7
Lung	23	0.16	26	25	0.07	47
Spleen	30	0.12	33	33	0.18	23
Muscle	5	0.94	5	4	0.03	>95%
Tumor	0	0.98	5	0	1.00	28
Pancreas	4	0.83	5	5	0.19	21
Sal. gland	22	0.00	>95%	22	0.01	>95%
Blood	1	0.96	5	1	0.39	11
Stomach	18	0.78	5	25	0.04	>95%
Sm. Int.	23	0.34	13	21	0.05	>95%
Lg. Int.	77	0.00	>95%	78	0.00	>95%

* P values are from a two-tailed student's t-test and the powers are calculated at a confidence level $\alpha=5\%$.