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^{99m}Tc-labeled nucleotides as tumor-seeking radiodiagnostic agents

(tumor imaging/diadenosine 5′,5‴,P^1,P^4-tetraphosphate and ATP/selective permeability)

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ABSTRACT **Several lines of human tumor cells in monolayer and soft agar cultures allow permeation of low levels of adenine nucleotides through their plasma membranes, while, in general, untransformed cells do not incorporate adenine nucleotides into their cellular pools without prior degradation of the nucleotides to adenosine. This study determined the uptake of ^{99m}Tc-radiolabeled chelated forms of adenine nucleotides, ^{99m}Tc-Ap$_4$A (diadenosine 5′,5‴,P^1,P^4-tetraphosphate) and ^{99m}Tc-ATP chelates as radiodiagnostic agents suitable for the *in vivo* detection of tumors by radionuclide imaging. Biodistribution studies revealed that Ap$_4$A accumulated preferentially in RT-24 tumors implanted in rats and that V2 carcinoma implanted in rabbits could be readily visualized by *in vivo* imaging. The biodistribution at various time points showed increased tumor-to-muscle ratios after ^{99m}Tc-Ap$_4$A or ^{99m}Tc-ATP injections when compared with a nonspecific marker of the extracellular fluid space, ^{99m}Tc-labeled diethylenetriaminepentaacetic acid and with an agent known to localize in some tumors, ^{67}Ga-labeled citrate. Studies of ectoenzymatic activities of virus-transformed animal cells and their untransformed counterparts in monolayer cultures showed marked decreases in the ectoenzymatic activities that degrade Ap$_4$A in the transformed cells. Incorporation of *en bloc* [^{3}H, ^{32}P]Ap$_4$A into cellular acid-soluble nucleotide pools of certain transformed cells was observed. Normal untransformed cells incorporated the radioactive label only by prior degradation to [^{3}H]adenosine and ^{32}P$_i$.**

It has recently been shown that certain human tumor cells are permeable to low levels of exogenous ADP and ATP through their plasma membranes and incorporate intact ADP and ATP into their cellular pools (1). When cells are grown in culture, the increased intracellular levels of these adenine nucleotides result in an arrest of cellular growth and cause a large fraction of the cells to remain in their S phase (1). Treatment of well-characterized human tumor cells in soft agar culture with 5–20 μM ATP yielded substantial inhibition of cellular growth (2).

Normal mammalian cells are usually impermeable to nucleotides in the surrounding medium (3, 4). Nevertheless, mammalian cells can be infected with low efficiency by viral nucleic acids (5), and mouse fibroblasts (L cells) were shown to be permeable to intact dAMP and araAMP (6), as well as other deoxynucleoside monophosphates (7). Incorporation of low concentrations of nucleotides into cellular pools is impaired by the activity of ectoenzymes such as ATPase, ADPase, alkaline phosphatase, 5′-nucleotidase, and nucleotide pyrophosphatase (8, 9). These enzymes are integral plasma membrane proteins with an active site situated on the outside of the cell (10). It has been reported that the activity of these enzymes fluctuates with the degree of cellular proliferation and differentiation (8).

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The permeability of tumor cells to intact adenine nucleotides could provide a diagnostic marker useful for the *in vivo* localization of tumors if an analog could be labeled with a useful gamma-emitting radionuclide. ^{99m}Tc is widely used for radionuclide imaging in humans because of its desirable physical properties: ^{99m}Tc is metastable, has a 6-hr physical half-life, and has an 85% incidence of a detectable gamma photon at 140 keV. These physical properties, coupled with scintillation cameras that are optimized for the 140 keV energy of ^{99m}Tc, make ^{99m}Tc the preferred radionuclide for scintillation imaging in humans (11). As a result of its desirable properties, ^{99m}Tc was selected as the radionuclide for labeling Ap$_4$A.

These studies were undertaken to determine the biodistribution of ^{99m}Tc-labeled Ap$_4$A (^{99m}Tc-Ap$_4$A) in experimental tumor-bearing animals and to test whether external imaging with a scintillation camera would permit localization of tumors *in vivo*.

MATERIALS AND METHODS

Materials. Nucleotides were purchased from Sigma and P-L Biochemicals. [^{3}H, β,γ-^{32}P]Ap$_4$A was prepared synthetically from AMP-imidazolylphosphinate and [^{3}H, γ-^{32}P]ATP (New England Nuclear) of specific activities 20 Ci/mmol for [^{3}H]ATP and 10 Ci/mmol for [γ-^{32}P]ATP (1 Ci = 37 GBq), following our published procedure (12).

Cells. 3T3 (BALB/c mouse fibroblast), SV-3T3 (simian virus 40-transformed 3T3 cells), BHK 21/13 (baby hamster kidney 21/13), Py-BHK 21/13 (polyoma virus-transformed BHK 21/13), SV-BHK, and C3A (human hepatoma) cells were cultured in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal bovine serum. Chicken embryo fibroblasts were cultured in DME medium supplemented with 5% fetal bovine serum/10% tryptose phosphate broth. Preparation of primary chicken embryo fibroblasts and their infection with Rous sarcoma virus were carried out according to published procedures (13).

Analytical Procedures. Nearly confluent cultures in 60-mm-diameter Petri dishes were washed 3 times with DME medium without serum and incubated at 37°C with [^{3}H, ^{32}P]-Ap$_4$A in 1.5 ml of DME medium without serum (prewarmed to 37°C). Ectoenzymatic activities of the cells catalyzing the degradation of [^{3}H, ^{32}P]Ap$_4$A were determined by thin-layer chromatography of 10 μl of the medium on poly(ethyleneimine)-cellulose (PEI-cellulose) thin-layer plates. Aliquots of the medium were spotted along with unlabeled markers of ATP, Ap$_4$A, ADP, AMP, and adenosine. The plates (20 cm long with a 5-cm attached paper wick) were developed with water and then 1 M LiCl. The spots corresponding to the unlabeled carriers, visualized by their ultraviolet light absorption, were cut out, eluted with 4 M NH$_4$OH, and counted in Bray's scintillation fluid.

Abbreviations: Ap$_4$A, diadenosine 5′,5‴,P^1,P^4-tetraphosphate; DTPA, diethylenetriaminepentaacetic acid.

Table 1. Distribution of ^{99m}Tc radioactivity in tissues of RT-24 gliosarcoma tumor-bearing rats after intravenous injection of ^{99m}Tc-Ap$_4$A

Organ	% injected dose per g of tissue				
	5 min	30 min	60 min	120 min	24 hr
Blood	1.49 ± 0.18	1.88 ± 0.18	0.73 ± 0.09	1.13 ± 0.11	0.12 ± 0.04
Brain	0.08 ± 0.01	0.03 ± 0.02	0.06 ± 0.01	0.02 ± 0.01	0.02 ± 0.03
Liver	4.05 ± 0.51	5.59 ± 0.47	3.02 ± 0.61	4.61 ± 0.64	4.09 ± 0.36
Heart	0.43 ± 0.09	0.22 ± 0.06	0.29 ± 0.12	0.17 ± 0.06	0.03 ± 0.02
Lung	0.84 ± 0.09	0.52 ± 0.08	0.46 ± 0.06	0.32 ± 0.04	0.12 ± 0.03
Kidney	7.89 ± 1.85	9.20 ± 0.49	10.79 ± 0.27	10.91 ± 0.69	6.41 ± 1.43
Bone	0.41 ± 0.08	0.13 ± 0.04	0.40 ± 0.06	0.08 ± 0.02	0.12 ± 0.03
Muscle	0.16 ± 0.04	0.04 ± 0.01	0.09 ± 0.01	0.03 ± 0.01	0.01 ± 0.01
Tumor	0.49 ± 0.11	0.14 ± 0.01	0.37 ± 0.12	0.08 ± 0.01	0.12 ± 0.03
Tumor/ muscle ratio	2.99	3.29	4.10	2.79	12.00

Values represent mean ± SD. n = 6.

After incubation for 2 min with [^{3}H, ^{32}P]Ap$_4$A in 1.5 ml of DME medium without serum at 37°C, the incorporation of [^{3}H, ^{32}P]Ap$_4$A into cells in monolayer cultures in 60-mm-diameter Petri dishes was assayed. The cells were washed 3 times with DME medium before the incubation and rapidly rinsed with 2.5 ml of DME medium after incubation. Cellular acid-soluble nucleotides were extracted as described (14). Chromatography was carried out on PEI-cellulose and the ^{3}H and ^{32}P radioactivities of cellular ATP were determined (14).

Preparation of ^{99m}Tc-Ap$_4$A. To couple the ^{99m}Tc to Ap$_4$A, various concentrations of Ap$_4$A (1–10 mg) were dissolved in 1 ml of saline and added to a solution of technetium pertechnetate. One milliliter of $SnCl_2$ solution (25 mg in 25 ml of 0.01 M HCl) was added to reduce the ^{99m}Tc, and the solution was mixed under a nitrogen atmosphere for 5–15 min. For the higher Ap$_4$A concentration of 10 mg, 3 ml of $SnCl_2$ solution was added. The mixture was then separated on a Sephadex G-25 column (2 cm long, 0.8 cm diameter). The product was eluted with saline into vials in ≈40% yield for the lower concentration and >60% for the higher concentration. The product was tested for its purity on TLC plates developed with saline and acetone.

^{99m}Tc-Labeled Diethylenetriaminepentaacetic acid (^{99m}Tc-DTPA) Preparation. A commercially prepared kit of lyophilized DTPA, stannous chloride, and buffers was purchased (New England Nuclear) checked for purity by TLC, and prepared as described by the manufacturer. Pertechnetate (2 mCi) was added to the kit and the solution was diluted with saline to the necessary volume for injection into the rats.

^{67}Ga-Labeled Citrate (^{67}Ga-Citrate). ^{67}Ga-citrate was purchased (New England Nuclear) and used without further preparation.

^{99m}Tc-Labeled ATP (^{99m}Tc-ATP) Preparation. Ten milligrams of ATP and 1 ml of $SnCl_2$ solution (3 mg in 1 ml of 0.01 M HCl) were mixed with 2–3 mCi of ^{99m}Tc-pertechnetate. The reaction mixture was kept under a nitrogen atmosphere for 5–15 min and was separated on a Sephadex G-25 column as described above with a >80% yield. The product was tested for purity on silica gel TLC plates developed with saline and acetone.

^{99m}Tc-Pertechnetate. This solution was obtained from a commercial ^{99}Mo-^{99m}Tc generator (New England Nuclear).

Rat Biodistribution. The biodistribution of ^{99m}Tc-Ap$_4$A, -ATP, and -DTPA and ^{67}Ga-citrate was studied in groups of six rats at each time point for each radionuclide. The rats (200–300 g CD strain Fisher rats) received 2–5 μCi of ^{99m}Tc-labeled agent in 0.1 ml via the tail vein. At 5, 30, 60, 120 min, and 24 hr after tracer administration, groups of animals were anesthetized, killed, and the entire heart, lungs, liver, spleen, kidney, bone, muscle, and tumor and the site of injection was dissected out, weighed, placed in scintillation vials and assayed for ^{99m}Tc or ^{67}Ga. The ^{99m}Tc was counted with a center line of 140 keV width and a 20% window; ^{67}Ga was counted with a single wide window ranging from a base line of 80 keV to an upper level of 350 keV. The percent injected dose per gram of tissue and percent injected dose per organ were calculated for each tracer.

Imaging Studies. The imaging properties of ^{99m}Tc-labeled agents were compared in rabbits implanted in the thigh with V2 carcinoma. The imaging studies were recorded with an all-purpose low-energy parallel hole collimator on an NSI upgraded Pho Gamma III scintillation camera (12.5-mm-thick crystal with 19 photomultiplier tubes, intrinsic resolution of 4.3 mm FWHM). The scintillation camera was interfaced to a nuclear medicine computer system to record the data. Sequential images were recorded for 10 min each at 1 hr and 24 hr after intravenous injection of the radionuclides.

Table 2. Distribution of ^{99m}Tc radioactivity in tissues of RT-24 gliosarcoma tumor-bearing rats after intravenous injection of ^{99m}Tc-Ap$_4$A

Organ	% injected dose per organ				
	5 min	30 min	60 min	120 min	24 hr
Blood	19.70 ± 2.59	25.58 ± 1.63	9.82 ± 0.83	16.18 ± 1.37	1.61 ± 0.54
Brain	0.12 ± 0.02	0.05 ± 0.03	0.08 ± 0.01	0.03 ± 0.01	0.02 ± 0.04
Heart	0.24 ± 0.04	0.13 ± 0.04	0.17 ± 0.07	0.10 ± 0.04	0.02 ± 0.01
Lung	0.77 ± 0.23	0.54 ± 0.06	0.42 ± 0.04	0.34 ± 0.04	0.10 ± 0.03
Kidney	12.78 ± 3.00	14.63 ± 0.61	17.40 ± 0.77	18.30 ± 0.46	9.23 ± 1.74
Bone	6.75 ± 1.38	2.13 ± 0.73	6.73 ± 0.88	1.45 ± 0.34	2.06 ± 0.51
Muscle	12.15 ± 2.99	3.24 ± 0.64	6.77 ± 0.76	2.31 ± 0.76	0.83 ± 0.51
Tumor	0.22 ± 0.14	0.51 ± 0.16	0.15 ± 0.04	0.38 ± 0.07	0.40 ± 0.12

Values represent mean ± SD. n = 6.

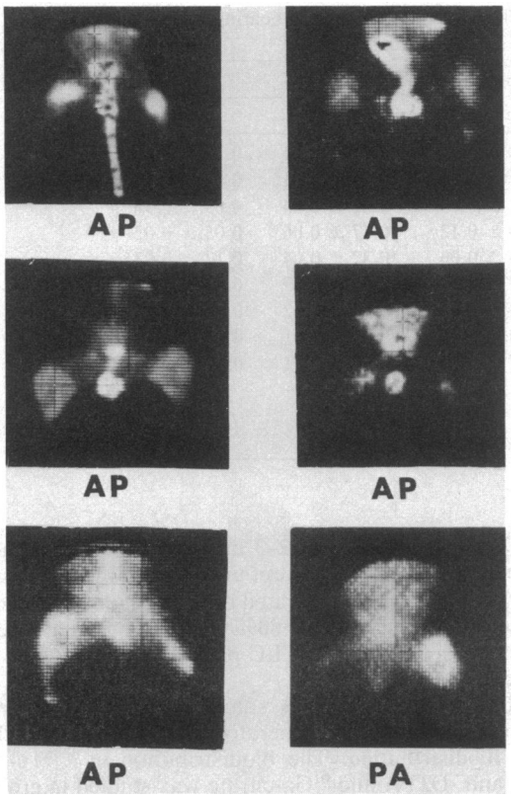

FIG. 1. Scintillation camera images 1 hr (*Left*) and 24 hr (*Right*) after injection of rabbits with V2 carcinoma implanted in the thigh. (*Upper*) ^{99m}Tc-Ap$_4$A (tumors in both right and left thigh); (*Middle*) ^{99m}Tc-DTPA (tumor implanted in right and left thigh); (*Lower*) ^{67}Ga-citrate (tumor in right thigh only). The 24-hr Ga image was recorded in the posterior position. AP, anterior posterior; PA, posterior anterior.

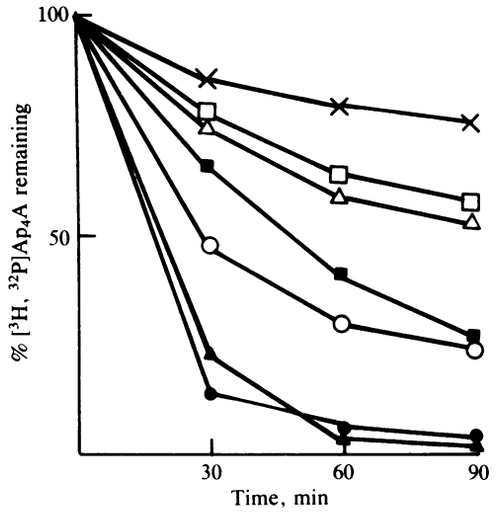

FIG. 2. Degradation of exogenous [^{3}H, ^{32}P]Ap$_4$A catalyzed by ectoenzymatic activities of cultured cells. Duplicate samples of 10^6 cells in culture were incubated at 37°C with 100 pmol of [^{3}H, ^{32}P]Ap$_4$A in 1.5 ml of DME medium without serum. The level of intact [^{3}H, ^{32}P]Ap$_4$A remaining in the medium was determined. ▲, BALB/c 3T3; △, SV-3T3; ■, BHK 21/13; □, SV-BHK; ×, Py-BHK; ●, chicken embryo fibroblast; ○, Rous sarcoma virus-transformed chicken embryo fibroblast.

RESULTS

Biodistribution of ^{99m}Tc-Ap$_4$A, -ATP, and -DTPA and ^{67}Ga-Citrate. Tables 1 and 2 summarize the biodistribution in percent dose per gram and percent dose per organ of ^{99m}Tc-Ap$_4$A as a function of the time after injection. The RT-24 gliosarcoma tumors implanted in rats exhibited an uptake of 0.49% dose per gram at 5 min and about 0.12% dose per gram in 24 hr. However, the tumor-to-muscle ratio was about 3:1 at 5 min increasing to 12:1 at 24 hr because of the faster clearance of the radiopharmaceutical from the blood as compared with the tumor. The tumor-to-blood ratio increased from 0.3:1 to 1:1 at 24 hr. These results are consistent with the images obtained in a rabbit with V2 carcinoma implanted in the thigh (Fig. 1). Quantification of the images revealed a tumor-to-muscle ratio of 3:1 at 1 hr. This ratio increased to 5:1 at 2 hr and remained at 5:1 after 24 hr. The results obtained with ^{99m}Tc-ATP, ^{99m}Tc-DTPA, and ^{67}Ga-citrate in tumor-bearing rats for 60 min and 24 hr are summarized in Table 3. The kidney activity was higher at all times with ^{99m}Tc-ATP and ^{99m}Tc-Ap$_4$A as compared to ^{67}Ga-citrate and ^{99m}Tc-DTPA.

The biodistribution of ^{99m}Tc-ATP and ^{99m}Tc-Ap$_4$A was similar in all tissues studied and different from the reference agent ^{99m}Tc-DTPA. The labeled DTPA had a lower blood level, kidney accumulation, and faster clearance from the tumor area than labeled Ap$_4$A. The behavior of ^{99m}Tc-Ap$_4$A is somewhat superior to ^{99m}Tc-ATP when the tumor-to-muscle ratios are compared; however, both agents showed preferential incorporation into the tumor tissue when compared to ^{99m}Tc-DTPA.

Typical images obtained with ^{99m}Tc-Ap$_4$A, -DTPA, and ^{67}Ga-citrate in a rabbit with V2 carcinoma implanted in the thigh are presented in Fig. 1. The active peripheral area of the tumor is delineated when compared to the ischemic center.

Degradation of [^{3}H, β,γ-^{32}P]Ap$_4$A by Ectoenzymatic Activities of Monolayer Cultures. Extracellular [^{3}H, ^{32}P]Ap$_4$A is rapidly degraded by the membrane proteins of a variety of

Table 3. Comparison of the tissue distribution of radioactivity in tumor-bearing rats 60 min and 24 hr after intravenous injection of the labeled agents

Labeled compound	Time after injection	% injected dose per g of tissue				Tumor/muscle ratio
		Blood	Kidney	Muscle	Tumor	
^{99m}Tc-DTPA	60 min	0.29 ± 0.10	1.50 ± 0.20	0.08 ± 0.06	0.21 ± 0.05	2.6
	24 hr	0.04 ± 0.01	0.49 ± 0.14	0.05 ± 0.01	0.02 ± 0.01	0.4
^{99m}Tc-ATP	60 min	1.09 ± 0.1	5.4 ± 0.57	0.16 ± 0.02	0.35 ± 0.05	2.2
	24 hr	0.33 ± 0.08	8.08 ± 2.48	0.05 ± 0.02	0.21 ± 0.08	3.5
^{67}Ga-citrate	60 min	2.07 ± 0.16	1.02 ± 0.13	0.27 ± 0.05	0.73 ± 0.26	2.7
	24 hr	0.24 ± 0.04	1.00 ± 0.13	0.25 ± 0.10	0.89 ± 0.12	3.6
^{99m}Tc-AP$_4$A	60 min	0.73 ± 0.09	10.79 ± 1.27	0.09 ± 0.01	0.34 ± 0.12	4.1
	24 hr	0.12 ± 0.04	6.41 ± 1.43	0.01 ± 0.01	0.12 ± 0.03	12.0

Values represent mean ± SD. n = 6.

Table 4. Incorporation of ^3H and ^{32}P radioactivity from exogenous [^3H, β,γ-^{32}P]Ap_4A into cellular ATP pools

Cell line	^3H cpm	^{32}P cpm
3T3	380	None
SV-3T3	450	None
BHK 21/13	870	None
Py-BHK	2,260	1,700
CEF	390	None
RSV-CEF	340	None
C3A (human hepatoma)	4,450	2,120

[^3H, β,γ-^{32}P]Ap_4A (100 pmol) was incubated for 2 min in 1.5 ml of DME medium without serum with 10^6 cultured cells at 37°C. Cellular [^3H, ^{32}P]ATP was analyzed as described. The $^{32}P/^3H$ ratio of the exogenous [^3H, ^{32}P]Ap_4A was 1.5. Data represent the mean of two experiments.

cell lines in monolayer cultures, as shown in Fig. 2. Untransformed cells possess higher levels of ectoenzymatic activities (nucleotide pyrophosphatase and phosphodiesterase), which catalyze the degradation of Ap_4A, than their virally transformed counterparts. In the case of chicken embryo fibroblasts, transformation of primary cultures with Rous sarcoma virus leads to a marked decrease of these ectoenzymatic activities 48 hr after infection.

Incorporation of [^3H, β,γ-^{32}P]Ap_4A into Cellular ATP Pools. Two minutes of incorporation of exogenous [^3H, β,γ-^{32}P]Ap_4A into monolayer cultures at 37°C was followed by assays of cellular [^3H, ^{32}P]ATP pools. The conversion of [^3H, ^{32}P]Ap_4A into [^3H, ^{32}P]ATP by intracellular metabolism minimizes the possibility of [^3H, ^{32}P]Ap_4A being adsorbed to the cells without permeation and incorporation into intracellular acid-soluble nucleotide pools. The 2-min incorporation period is not sufficient to produce detectable extracellular [^3H, ^{32}P]ATP formed by the extracellular degradation of [^3H, ^{32}P]Ap_4A. In monolayer cultures (Table 4) only Py-BHK and C3A (human hepatoma) cells allow permeation of [^3H, ^{32}P]Ap_4A and its incorporation into their intracellular pools. These two cell lines exhibit much lower ectoenzymatic activities, which degrade extracellular [^3H, ^{32}P]Ap_4A, in comparison to the untransformed cell lines that did not allow permeation of [^3H, ^{32}P]Ap_4A through their plasma membrane. We thus conclude that the intracellular [^3H, ^{32}P]ATP was produced by *en bloc* incorporation of [^3H, ^{32}P]Ap_4A into intracellular pools followed by intracellular metabolism. Incorporation due to prior degradation of [^3H, ^{32}P]Ap_4A into [^3H]adenosine and $^{32}P_i$ results in identification of only [^3H]ATP in the intracellular pools because of dilution of $^{32}P_i$ by the high concentrations of inorganic phosphate in the medium and by intracellular P_i pools. The data presented in Table 4 indicate that [^3H, ^{32}P]ATP can be identified in the intracellular pools of Py-BHK and C3A (human hepatoma) cells; all other cell lines tested yield [^3H]ATP, which suggests degradation of [^3H, ^{32}P]Ap_4A to [^3H]adenosine before incorporation into intracellular acid-soluble nucleotide pools.

DISCUSSION

These studies confirm that certain transformed animal cells in culture contain lower levels of ectoenzymatic activities, which catalyze the degradation of acid-soluble adenine nucleotides than do their untransformed counterparts, and that some, but not all, transformed animal and human cells in culture allow incorporation of low levels of intact adenine nucleotides into their intracellular pools. The negative charges of the phosphate moieties can contribute importantly to the lack of intracellular penetration (15). It is important to note that nonspecific phosphomonoesterase activities of human tissues were found to be much lower than the same activities of comparable mouse tissues (16, 17).

We have capitalized on the properties of tumor cell membranes that allow permeation of acid-soluble nucleotides without their prior degradation in using ^{99m}Tc chelated to Ap_4A as a radiodiagnostic agent for tumor imaging in live animal models, and we compared its behavior to other agents such as ^{99m}Tc-ATP, ^{99m}Tc-DTPA, and ^{67}Ga-citrate. ^{67}Ga-citrate is presently used as an effective tumor- and abscess-localizing agent in nuclear medicine procedures (18, 19) and ^{99m}Tc-DTPA represents a gamma emitter chelated to a nonspecific chelator (DTPA). The data reported here indicate that ^{99m}Tc-labeled adenine nucleotides produce preferential tumor-seeking agents with a desired biodistribution in live animal models.

The accumulated activity of Ap_4A labeled with a gamma emitting agent in tumors implanted in live animals suggests a potential use of labeled nucleotides for *in vivo* tumor detection and for the assessment of tumor behavior after therapy. The high accumulation of radioactivity in the kidney may be useful for visualization of intrarenal or perirenal tumors. Further investigations will be necessary to determine whether ^{99m}Tc is still chelated to the nucleotides in the kidney, because the latter organ is well known for its excretory function in relation to heavy metals.

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