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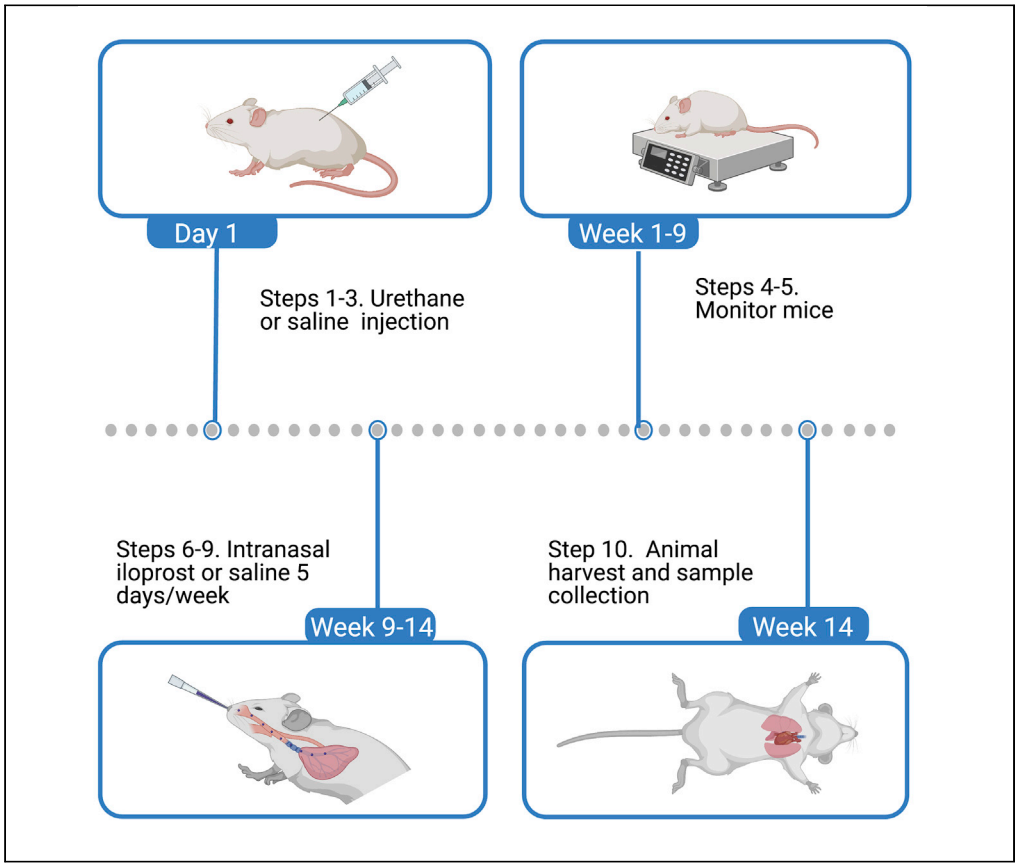
Protocol for intranasal chemoprevention delivery in a urethane mouse lung cancer model



Mouse iloprost lung cancer chemoprevention studies typically use oral delivery. Here, we present a protocol for intranasal iloprost delivery within a urethane lung adenocarcinoma mouse model. We detail steps for intraperitoneal urethane injection in mice, followed by nine-week monitoring, intranasal iloprost treatment, and lungs harvesting for analysis. This iloprost delivery approach parallels an ongoing phase II clinical trial of inhaled iloprost for lung cancer chemoprevention. This protocol diversifies options for chemoprevention studies and offers a relevant and translatable model.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Lori Dwyer-Nield, Robert L. Keith, Meredith A. Tennis

meredith.tennis@cuanschutz.edu

Highlights

Intranasal delivery of iloprost chemoprevention in a urethane lung cancer mouse model

Single urethane injection and regular intranasal iloprost treatment for five weeks

Lung harvest and analysis at 14-week time point

Approach applicable to other inhaled lung cancer chemoprevention agents

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Protocol for intranasal chemoprevention delivery in a urethane mouse lung cancer model

Lori Dwyer-Nield,[1] Robert L. Keith,[2,3] and Meredith A. Tennis[3,4,5,*]

[1]University of Colorado School of Pharmacy, Aurora, CO 80045, USA

[2]Rocky Mountain Regional VA Medical Center, Aurora, CO 80045, USA

[3]University of Colorado School of Medicine, Aurora, CO 80045, USA

[4]Technical contact

[5]Lead contact

*Correspondence: meredith.tennis@cuanschutz.edu
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SUMMARY

Mouse iloprost lung cancer chemoprevention studies typically use oral delivery. Here, we present a protocol for intranasal iloprost delivery within a urethane lung adenocarcinoma mouse model. We detail steps for intraperitoneal urethane injection in mice, followed by nine-week monitoring, intranasal iloprost treatment, and lungs harvesting for analysis. This iloprost delivery approach parallels an ongoing phase II clinical trial of inhaled iloprost for lung cancer chemoprevention. This protocol diversifies options for chemoprevention studies and offers a relevant and translatable model.

For complete details on the use and execution of this protocol, please refer to Sompel et al. (2022).

BEFORE YOU BEGIN

Institutional permissions

All studies with animals must be conducted in accordance with the Guide for Care and Use of Laboratory Animals and approved by the appropriate institutional IACUC committee. An SOP for urethane must also be in place to protect humans handling urethane injections or exposed mice.

All described experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals. All experiments were approved by the Rocky Mountain VA Medical Center Veterinary Medical Unit IACUC and conducted with an SOP for use of urethane in animal studies.

Mice

Timing: Depends on animal facility process

1. Order 8–10 week old, female mice.
   a. Allow mice to acclimate for two weeks before beginning the experiment.
   b. Mice should be maintained on hardwood bedding in a 12 h light/dark cycle with a standard mouse lab diet and water ad libitum.
   c. The number of mice depends on study endpoints and whether multiple samples will be collected from a single mouse, but the chemoprevention protocol typically requires group sizes of 7–10 to achieve significance.

Note: Urethane can be used as a carcinogen in many strains of mice but intranasal iloprost has only been tested in the FVB/N and A/J strains. Selection of strain should be based, when

possible, on previous studies of the carcinogen and chemoprevention agent. Female FVB/N mice are preferred due to aggression associated with increased handling of male mice. Different strains of mice may have different behavior patterns and both sexes could be used. Susceptibility to urethane varies between strains but resistance to urethane can be overcome with increased urethane injections (Miller et al., 2003).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Urethane	Sigma-Aldrich	943-50g
Iloprost	Cayman Chemical	18215
Saline (sterile, pharmaceutical grade)	Fisher Scientific	Z1376
Oxygen	Gas supplier	
Isoflurane anesthetic	Pivetal	21295097
Fatal Plus	Vortech Pharmaceuticals	0298-9373-68
Experimental models: Organisms/strains		
FVB/N mice; wild type; 8–10 weeks old; male and female	Charles River	207
Other		
0.22 μm syringe filters	Fisher Scientific	09-720-3
15 mL Conical tubes	Fisher Scientific	S50712
Mouse scale	Fisher Scientific	01-913-933
5 mL sterile syringes	Fisher Scientific	14-955-452
Gas anesthesia delivery system	Kent Scientific	SF-MSEKIT
p200 pipette and sterile filter tips	Fisher Scientific	S98636A, 02-707-478
2 mL microcentrifuge tubes	Fisher Scientific	05-408-138
70% alcohol swabs	Fisher Scientific	S17032
1 mL sterile syringes with needle (25 g × 5/8)	Fisher Scientific	14-817-125
Biosafety Cabinet	Fisher Scientific	S35894
Dissecting board and pins	Fisher Scientific	14-370-284, S99385
Mouse surgical sterile tool kit	Kent Scientific	INSMOUSEKIT
Tools for sample collection (depends on type collected)	N/A	N/A
Tissue/sample collection containers (depends on type collected)	N/A	N/A

⚠ CRITICAL: Urethane is a hazardous chemical and must have an SOP for use with animals. Urethane exposure can lead to birth defects, eye irritation, respiratory damage, and skin damage and can affect the liver, bone marrow, and reproductive system. Cages with urethane exposed mice should be identified and all work with urethane or urethane exposed animals should be performed in a fume hood or safety cabinet. Bedding should be disposed of in biohazard trash. Mask, gown, eye protection, and gloves should be used while working with urethane. Isoflurane is a halogenated anesthetic gas used to anesthetize research animals. Waste anesthetic gases must be properly scavenged or exhausted. Short-term overexposure may result in irritation of the eyes, skin and respiratory tract, cough, sore throat, and may impair consciousness and motor skills. Overexposure can lead to adverse effects on the liver, kidneys, and reproductive system.

Alternatives: Other intranasal administered chemoprevention agents can be used with this protocol but should be optimized for dose and delivery timing. Intranasal iloprost could be used to prevent lesions induced by other carcinogens but should be optimized for timing when combined with other protocols.

STEP-BY-STEP METHOD DETAILS

Urethane injection

Timing: 2 h (depending on number of mice); injections completed in the morning

In this step, urethane is injected to initiate the carcinogenesis process. Saline injections are used in control animals. FVB/N or A/J mice are administered with one intraperitoneal (IP) injection of 1 mg urethane/g body weight dissolved in 0.9% saline.

1. Weigh mice and record weights.
2. Prepare urethane.
   a. Stock urethane is stored at 20–25°C for up to two years. Urethane should be diluted the morning of the injections and can be stored at 20–25°C for eight hours.
   b. Use the table below to determine the required volume and concentration of urethane solutions based on mouse weights and number of mice in each weight category.
   c. Weigh urethane calculated from the table into pre-tared tubes.
   d. Add sterile saline and vortex to dissolve urethane.
   e. Sterile filter solution into new sterile tubes.

Number of mice to be injected	10	20	30	40
Weight (grams) of mouse	mg of Urethane			
16 (15.0–16.9)	160	320	480	640
18 (17.0–18.9)	180	360	560	680
20 (19.0–20.9)	200	400	560	720
22 (21.0–22.9)	220	440	600	760
24 (23.0–24.9)	240	480	640	800
26 (25.0–26.9)	260	520	680	840
28 (27.0–28.9)	280	560	720	880
30 (29.0–30.9)	300	600	760	920
32 (31.0–32.9)	320	640	800	960
34 (33.0–34.9)	340	680	840	1,000
36 (35.0–36.9)	360	720	880	1,040
Total volume of prepared solution	1 mL	2 mL	3 mL	4 mL

3. Inject urethane or saline.
   a. Fill a new sterile syringe with needle (25 gauge, 5/8 inch) with 100 μL of the appropriate urethane concentration for each mouse according to its weight.
   b. Put mouse cages in a biosafety cabinet, remove a mouse to identify and verify its weight with recorded weights, swab abdomen with 70% alcohol, and inject urethane IP (Figure 1).
   c. For saline control injections, fill a new sterile syringe with needle with 100 μL sterile saline, remove a mouse to identify, swab abdomen with 70% alcohol, and inject IP.
   d. Return mice to cage and monitor hourly until fully recovered.

⚠ CRITICAL: Urethane is an anesthetic and mice should experience lethargy within minutes of injection that persists for 1–5 h. Mice do not lose the ability to right themselves or to maintain body heat but assess the mice for ability to right themselves before beginning hourly monitoring. Mice regain activity and show no negative effects of injection within a few hours. Any mice which become unresponsive or fail to recover should be euthanized, but this is an extremely rare event. Troubleshooting 1. Technical expertise with IP injections is key to consistent experimental results. Personnel should wear appropriate PPE when working with urethane or urethane exposed animals and their bedding and SOPs should be established with individual IACUCs.

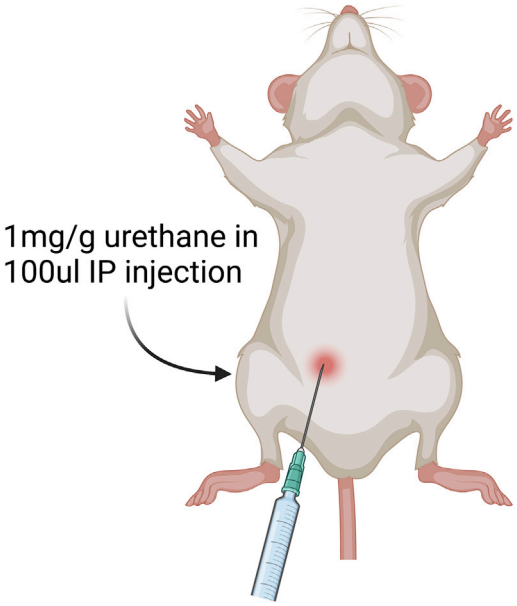

Figure 1. Urethane exposure
Urethane is delivered by a 100 μL IP injection as a 1 mg/g body weight dose to 8–10 week old mice. Control mice are injected IP with 100 μL saline. Mice become lethargic and are monitored during recovery but do not lose ability to right themselves or regulate body heat.

Note: Dissolving urethane in saline requires extensive mixing and care should be taken to ensure complete dissolution. Dosing for other strains of mice or other carcinogens should be determined experimentally or from published studies. Plan to deliver 1 mg/g body weight in 100 μL volume sterile saline to each mouse. For example, for 20 mice weighing 24 grams each, 480 mg of urethane would be dissolved in 2 mL of sterile saline. At the low dose of urethane used in this protocol, mice are lethargic for only a short time and do not lose the ability to right themselves or regulate body heat. Heated cages or heating pads can be used if animals need assisted recovery.

Monitor animals

Timing: For 14 weeks after urethane injection

Animals are monitored daily for the first week and weekly thereafter during lesion development for overall health. This step ensures humane treatment of the animals and can identify early in the study any major problems (Figure 2).

4. Weigh mice and record weights daily for the first week after urethane injection.
   a. Evaluate animals who approach 15% weight loss. Determine if nutritional support is appropriate.
   b. Euthanize animals who have more than 15% weight loss or are demonstrating sick rodent postures, abdominal distension, a black-hued abdomen, or difficulty breathing. Necropsy when indicated to determine cause of death.
5. Weigh mice and record weights weekly for weeks 2–14 after urethane injection.
   a. Evaluate animals who approach 15% weight loss. Determine if nutritional support is appropriate.
   b. Euthanize animals who have more than 15% weight loss or are demonstrating sick rodent postures, abdominal distension, a black-hued abdomen, or difficulty breathing. Necropsy when indicated to determine cause of death.

Note: Depending on the strain, some mice are more strongly affected by urethane toxicity and need additional nutritional support (treats) or monitoring. Before conducting carcinogen

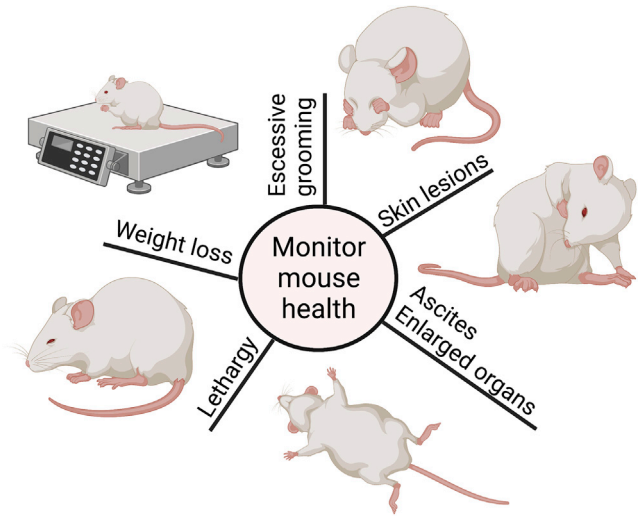

Figure 2. Mouse health monitoring

As with all chemical carcinogen protocols, mice are monitored closely for signs of declining health. For the first week after urethane injection, mice are weighed daily and evaluated for pain or distress. Thereafter and until sacrifice, mice are weighed weekly and evaluated daily for pain or distress. Weight loss is the most common sign of declining health in urethane studies.

studies, lab experience or the literature should be used to estimate the risk of off-target health effects in specific strains. Necropsies of animals euthanized before study endpoint may identify areas for technical improvement or susceptibilities in specific strains. Our recommended weight loss cutoff of 15% for humane euthanasia are included in the steps above and were established by our experience and preference of institutional veterinary staff. Investigators should establish weight loss thresholds with individual institutional IACUCs.

Administer intranasal iloprost

Timing: At week 9 after urethane injection; 1 h (to treat, depending on animal number)/day, 5 days/week, for 5 weeks

In this step, intranasal iloprost is administered as a chemopreventive agent to reduce development of premalignant lesions and carcinomas. Monitoring of overall animal health, as described above, continues throughout iloprost administration.

6. Prepare iloprost.
   a. Stock iloprost is stored at -20°C for up to one year. Diluted Iloprost should be prepared immediately before use at 20–25°C and not stored beyond the time required for administration.
   b. Dilute iloprost in sterile saline to achieve a concentration of 5 μg/100 μL and the volume needed for 100 μL per mouse.
   c. Use a sterile syringe and needle to collect iloprost from vial.
   d. Add sterile saline to a sterile tube at a volume of 100 μL per control mouse.
   e. Use a sterile syringe and needle (or other sterile approach) to collect saline from stock container.
7. Administer intranasal iloprost.
   a. Anesthetize mice with isoflurane (3% in oxygen) according to institutional guidelines.
   b. Once mice have stopped moving and are breathing slowly and deeply (3–4 min), load a p200 pipette with 100 μL iloprost or saline solution.
   c. Hold the mouse by its ears and tilt its head back. Deliver a drop of solution to the edge of the mouse's nare (Figure 3).

Figure 3. Intranasal iloprost administration
Iloprost is delivered nine weeks after the urethane injection. Mice are anesthetized with isolflurane (3% in oxygen) and 5 μg of Iloprost is delivered by two 50 μL doses, one to each nare of the mouse. Control mice are administered two 50 μL doses of saline. Treatments occur 5 days/week for five weeks. Recovery from the brief dose of anesthetic is quick and when mice are confirmed to be breathing normally and awakening, they are returned to their home cage.

d. Wait for the mouse to inhale the solution, then apply more until 50 μL have been dispensed.
e. Deliver the second 50 μL to the other nare.
f. Before returning the mouse to its cage, make sure it is breathing regularly. If not, tap on its chest or back or move it around gently.
g. Ensure mouse is waking up before returning cage to housing.

8. Repeat steps 6 and 7 to deliver iloprost five days per week for five weeks.
9. Monitor animals during iloprost treatment weeks according to urethane guidelines (step 5).

⚠ CRITICAL: Training and practice with intranasal delivery should be conducted with saline before an experiment is initiated to establish efficient and swift technique. Initial attempts may result in solution dripping into the mouth and being breathed in or swallowed through the mouth.

Optional: In this model, mice receive 5 μg of iloprost in 100 μL five days/week for five weeks. The combination of iloprost with other carcinogens or in different strains may require alternate dosing. Chemoprevention agents can be delivered in smaller volume depending on required concentration and solubility. Iloprost can be requested from Cayman Chemical in different formulations depending on experimental needs.

Note: The 5 day/week administration of 5 μg/100 mL iloprost/saline parallels dosing of iloprost in humans, but if needed, alternative dosing schedules that reduce animal-human contact could be tested for efficacy. Dosing for other strains of mice or other chemopreventive agents should be determined experimentally or from published studies. Concentrated agents may result in nasal irritation, so a pilot study should be conducted with new agents to ensure mice can tolerate the desired dose. Work quickly when delivering agents intranasally because mice quickly recover from their brief exposure to isoflurane. If intranasal iloprost administration is slow and mice are receiving longer delivery of gas anesthetic, heated cages or heating pads can be used to support recovery.

Study endpoint

Timing: 14 weeks after urethane (depends on experiment parameters); harvest time depends on sample collection needs

Hyperplasia and adenomas begin to appear 3–6 weeks after urethane injection in FVB/N mice. At 14–16 weeks, FVB/N have 3–6 adenomas >1 mm and adenocarcinoma appears after 30 weeks. This step counts lesions and collects samples for analysis. Standard protocols for lung lesion and tissue harvest have been previously published. (Sozio et al., 2021; Davenport et al., 2020) Exact protocols for harvest depend on the specific study and samples being collected.

10. Harvest lesions and tissue.

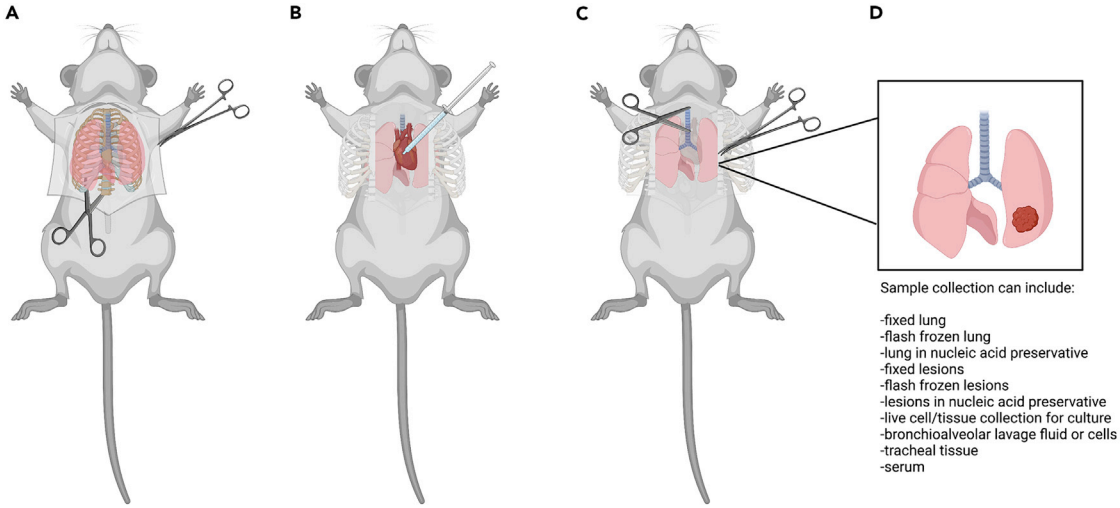

Figure 4. Sample collection

14 weeks after the urethane injection, mice are sacrificed by pharmacologic euthanasia and exsanguination.

(A) The thoracic cavity is opened.

(B) The rib cage is cut through the sternum and separated. The lungs are perfused with cold saline solution.

(C) The trachea is cut and connective tissue is separated to facilitate removal of the lungs.

(D) Lung, tracheal, and lesion tissue can be collected according to study needs.

a. Mice are injected IP with 200 mg/kg Fatal Plus and when animals are unresponsive to toe pinch, blood samples can be collected by cardiac puncture with a syringe and needle (25 g × 5/8) and processed for downstream analysis.
b. Death is confirmed by exsanguination and cessation of heartbeat and breathing in accordance with IACUC guidelines.
c. Spray mouse with 70% ethanol, cut open skin to expose thoracic and abdominal cavity, cut the inferior vena cava, and expose the lungs by cutting through the rib cage (Figure 4).
d. Perfuse the lungs with cold saline solution in a 5 mL syringe by inserting the syringe into the right heart ventricle. Perfusion is indicated by lung inflation and white color of the tissue.
e. Sample collection.
    i. Lungs can be inflated with 10% formalin for fixation and the trachea tied off before removal.
    ii. Alternatively, use a hemostat to clamp the left bronchus, remove the left lobe for fresh or frozen analysis, and then proceed with inflating the right lung with formalin.
    iii. Lobes can be flash frozen in liquid nitrogen, stored in nucleic acid preservative (RNA Later) for nucleic acid extraction, or processed for fresh tissue or cell analysis.
    iv. Alternatively, whole lung can be dissected by holding the trachea and gently removing connective tissue around the lungs, leaving them intact.
    v. Whole lung can be transferred to saline or formalin, depending on downstream analysis.
f. Troubleshooting 2, troubleshooting 3.

⚠ CRITICAL: Pharmaceutical overdose should be used for mouse euthanasia to protect airway and lung tissue from damage or physiological alterations (for example, Fatal Plus or ketamine/xylazine). Alternatively, CO_2 with cervical dislocation can be used for euthanasia.

Optional: Our experience is with harvesting lesions and tissues after urethane and iloprost at 14 weeks, but depending on the experimental plan, the harvest could be conducted at a later time point. If fixed tissue analysis is planned, the simplest approach is to add animals to each group for fixation of the whole lung. It is possible to tie off and remove the left lobe for biochemical analysis and fix only the right lobe, but if tumor counting is an endpoint, the

left lung should be counted immediately after removal and the right fixed lung should be serially sectioned at 5 μm and every tenth slide analyzed for lesions (about 15 slides per mouse).

EXPECTED OUTCOMES

A single urethane injection leads to an average of 4–6 adenomas in FVB/N and 20–30 in A/J after 14 weeks, which is reduced by intranasal iloprost treatment to an average of 2–3 (Sompel et al., 2022; Tennis et al., 2022).

QUANTIFICATION AND STATISTICAL ANALYSIS

To achieve statistical significance, groups of 7–10 are typically required. All lesion counts from the five lobes are combined and comprise lesion multiplicity for that mouse. Tumor size can also be recorded using a digital caliper. Histologic analysis of lung tissue can be done to identify hyperplasia, microadenoma, adenomas, and adenocarcinoma using imaging. Lesion morphology and grading should be determined by two independent, blinded reviewers. Mouse weights, differences in lesion number or size, or endpoint assays can generally be analyzed by one-way ANOVA with Tukey's post hoc analysis or a two-tailed Student's t-test. While not common, animals found to have significant lung disease (i.e., pneumonia) on observation of lung tissue or on H&E besides adenomas or tumors can be excluded from analysis as the comorbidity often affects relevant data points.

LIMITATIONS

While it is a well-established mouse lung carcinogen, use of urethane as a carcinogen can be affected by environmental conditions, mouse strain, or animal stress or undiagnosed illness, leading to unexpected rates of death or lack of tumor induction. Additional chemical lung carcinogens that have been used in chemoprevention studies include cigarette smoke, benzo(a)pyrene, and nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. (Keith et al., 2004; Kassie et al., 2022; Islam et al., 2022).

TROUBLESHOOTING

Problem 1

Lack of lethargy after urethane injection.

Potential solution

Lack of lethargy after urethane injection suggests incorrect injection technique, incorrect dosing, or compromised urethane (after step 2). If it is determined than an error in urethane preparation occurred, urethane can be prepared again, and animals reinjected the next day. Other sources of error should be corrected with training or a new lot of urethane before animals are injected again. To avoid compromised urethane, dispose of the reagent when it reaches two years old.

Problem 2

Lack of lesions at study endpoint.

Potential solution

FVB/N and A/J mice are well described as developing early adenomatous lesions at 16–20 weeks after urethane and adenocarcinoma by 30 weeks after urethane. If lesions are not present (step 7), the specific strain of mice may need more urethane, for example, C57/B6 mice require six injections of urethane to develop adenocarcinoma. Sensitivity to urethane has been described for many strains of mice (Miller et al., 2003; Titis and Forkert, 2001; Dwyer-Nield et al., 2010). Compromised urethane may also reduce lesion development.

Problem 3

Animal death before study endpoint.

Potential solution

In our experience, FVB/N mice may have up to 10% premature death rate from urethane after urethane injections. This occurs sporadically and the cause has not been identified. The toxicity appears as a distended and/or discolored abdomen about 4 days after urethane exposure, followed by weight loss requiring euthanasia. Other strains may experience less premature death, but some death is expected. Mice do not die or are euthanized early due to tumor development, as studies in chemoprevention conclude before any animal experiences significant tumor burden. Compromised urethane may lead to lack of response or toxicity, so we recommend replacing urethane within two years after purchase.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Meredith Tennis, Meredith.tennis@cuanschutz.edu.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

L.D.-N.: Conceptualization, methodology, review & editing. R.L.K.: Conceptualization, funding acquisition, review & editing. M.A.T.: Conceptualization, funding acquisition, writing, review & editing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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