

Screening of Chemopreventive Agents in Animal Models: Results on Reproducibility, Agents of a Given Class, and Agents Tested During Tumor Progression



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Abstract

Because of the importance of testing reproducibility of results, we present our findings regarding screening agents in preclinical chemoprevention studies in rodent models performed by the Chemopreventive Agent Development Research Group (CADRG) of the Division of Cancer Prevention of the NCI. These studies were performed via contracts to various commercial and academic laboratories. Primarily, results with positive agents are reported because positive agents may progress to the clinics. In testing reproducibility, a limited number of direct repeats of our standard screening assays were performed; which entailed initiating treatment shortly after carcinogen administration or in young transgenic mice and continuing treatment until the end of the study. However, three additional proto-

cols were employed relating to reproducibility: (i) testing agents at lower doses to determine efficacy and reduced toxicity; (ii) testing agents later in tumor progression when microscopic lesions existed and, (iii) testing multiple agents of the same mechanistic class. Data with six models that were routinely employed are presented: MNU-induced ER-positive mammary cancer in rats; MMTV-Neu ER-negative mammary cancers in transgenic mice; AOM-induced colon tumors in rats; intestinal adenomas in Min mice; OH-BBN-induced invasive rat urinary bladder cancers in rats; and UV-induced skin squamous carcinomas in mice. It was found that strongly positive results were highly reproducible in the preclinical models evaluated. *Cancer Prev Res*; 11(10); 595–606. ©2018 AACR.

Introduction

Perhaps the greatest (yet rarely asked) question for published biological science is "can the data be reproduced?" The possibility that a great deal of published science cannot be reproduced is disturbing. This question has been raised in various articles (e.g., 1, 2). Recently there have been initial reports from "The Reproducibility Project: Cancer Biology" which endeavored to reproduce the findings of 50 articles published in high impact journals (3). Most of the studies involved reflected important mechanistic questions, and the results of the reproducibility studies yielded "muddy

results" (3). The reproducibility questions raised by these reports have spurred the National Cancer Institute (NCI) to address the question of reproducibility in research grants (grants.nih.gov/reproducibility/index.htm). Data are presented in this review on the reproducibility of testing agents for chemopreventive activity in a variety of *in situ*-arising models (chemically induced or transgenic animal models). Theoretically, different types of reproducibility studies can be examined. One is to exactly reproduce the initial study that was performed. This can often be difficult when based on reports in the literature due to a lack of experimental details. However, because our screening assays with a given model are normally standardized, this was not as difficult.

Because the primary objective of the CARDG is to identify agents that may progress to the clinic, most of the agents examined were clearly positive. Although direct repeats of our initial studies were infrequent, more indirect studies of reproducibility were performed. These more indirect studies include testing the efficacy of multiple members of a class of agents, whether administration of the agent can be delayed, and testing the efficacy of lower doses of the agent. Although the primary objective of this

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review is to examine the reproducibility of studies performed in animal models, the results of the altered reproducibility testing shed light on three additional points: (i) multiple agents from the same mechanistic class have similar effects; (ii) a given agent, or class of agents, tends to be highly effective in specific organ sites, but not all organs; and (iii) most of the agents examined are effective when administered later during the tumor progression stage, and are not solely effective by inhibiting the very earliest stages of tumor progression. In assessing the efficacy of agents in later stages of progression, one must be aware that most animal tumors are not as advanced at the genomic level (amplifications, deletions) as most human tumors; and (iv) many of the agents show some efficacy at doses lower than the higher dose employed in our initial screen, which may make them useful in combination studies with other agents to reduce toxicity (this point, however, is not addressed in the current article).

This work examines the results of testing potential chemopreventive agents in multiple *in situ*-arising cancer models performed under the auspices of CADR. The objectives of these studies were to examine the ability of various agents (overwhelmingly individual chemical entities) to inhibit/prevent the development of cancers. Although the CADR has studied numerous models (4), we will present data with only six models (Table 1) that we routinely employ: two models of mammary cancer [methylnitrosourea (MNU)-induced estrogen receptor positive (ER⁺) cancer in rats and *Neu*-driven ER⁻ mammary cancer in a transgenic mouse]; two models of colon/intestinal cancer [azoxymethane (AOM)-induced colon cancer in F344 rats and intestinal adenomas in Min mice]; a single model of invasive urinary bladder cancer induced by hydroxylbutyl(butyl)nitrosamine (OH-BBN) in rats; and an ultraviolet (UV) light-induced model of squamous cell skin cancer in SKH hairless mice. For many of the models that we use less frequently, we cannot address this reproducibility question (4).

A few of the specific characteristics of this article are as follows: (i) as a screening effort for NCI, we were primarily looking for positive agents that might proceed to clinical trials. Thus, the data were likely to involve repeated studies with positive agents. Direct reproducibility data with a limited number of negative agents in the MNU model are also presented. In addition, we present a limited number of studies employing suboptimal doses of highly effective agents usually employed in drug combination protocols; (ii) typically our labs did not exactly reproduce the protocol of our initial chemoprevention study, even on positive compounds. However, we were likely to perform three protocols related to reproducibility. First, most phase III cancer prevention trials take place late in lesion progression (roughly 5 years to a cancer endpoint), and we felt that any agent that was highly active when agent administration was begun around the time of tumor initiation, or in a very

Table 1. Characteristics of the animal models employed

A. MNU-induced mammary cancer in Sprague-Dawley rats	Characteristics: Minimally invasive ER positive adenocarcinomas induced by methylnitrosourea in adolescent rats. Tumors by genomic analysis are similar to human well-differentiated ER positive breast cancers.
B. MMTV-<i>Neu</i>/P53ko transgenic mice develop <i>Neu</i> overexpressing ER negative mammary cancers	Characteristics: ER negative mammary carcinomas in MMTV- <i>Neu</i> transgenic mice. Tumors overexpress wild type <i>Neu</i> , and have altered P53 like human ER negative <i>Neu</i> over expressing tumors.
C. Min mice develop multiple intestinal lesions	Characteristics: Mice have germline mutation in the APC gene induced by the mutagen ENU. Mutations in APC are found in human with familial adenomatous polyposis syndrome and most sporadic colon polyps and cancers. The mice develop tumors (adenomas) primarily in the small intestine.
D. AOM-induced colon cancers in Fischer-344 rats	Characteristics: Colon tumors are induced by organ-specific carcinogen, azoxymethane (AOM). These are minimally invasive adenocarcinomas in colon. Tumors have mutations in β -catenin, the same pathway found in humans (APC gene) and roughly 40-50% of rat colon tumors have Ki Ras mutations like humans.
E. OH-BBN-induced invasive urinary bladder cancers in female Fischer-344 rats	Characteristics: Tumors induced by organ-specific carcinogen hydroxybutyl-butyl-nitrosamine yield invasive urinary bladder cancers. These tumors look histopathologically similar to human invasive bladder cancer. RNA analyses have shown substantial overlap between invasive human bladder cancer and those observed in the rodent bladder cancers.
F. UV-induced squamous cell skin cancers in SKH hairless mice	Characteristics: Squamous cell skin cancers induced by repeated UVB exposure. This is the same carcinogen as in humans. Histologically, tumors look like those in humans. Mouse and human SCCs driven by P53 mutations at dipyrindine sites.

Abbreviations: AOM, azoxymethane; APC, Adenomatous polyposis coli; ENU, Ethylnitrosourea; ER, estrogen receptor; MMTV, mouse mammary tumor virus; MNU, *N*-methyl-*N*-nitrosourea; RNA, ribonucleic acid; SCC, Squamous cell carcinoma.

young transgenic animal, then that agent should also be examined in a delayed setting, for example, in colon after aberrant crypt foci (ACF) or early lesions already existed (5), or in urinary bladder after microinvasive lesions (6) had arisen. Second, if an agent is highly effective at all doses tested (typically two) in our primary screen, we often tested it at multiple doses, including significantly lower ones; at least in part to determine a dose to employ in a combination protocol using two agents. Third, and the slightly more indirect measure of reproducibility (albeit, perhaps the most important practically) is testing multiple agents of a specific mechanistic class in each cancer model. Testing multiple NSAIDs (piroxicam, sulindac, ibuprofen, naproxen, nimesulide), all of which are both COX-1 and COX-2 inhibitors, gave us an indirect test of reproducibility. Similarly, testing multiple selective ER modulators, or SERMs, (tamoxifen, toremifene, arzoxifene, bazedoxifene) in the MNU-induced breast model, or multiple EGFR inhibitors (gefitinib, erlotinib, lapatinib) in urinary bladder or mammary cancer models, are indirect reproducibility tests. These class-specific studies are particularly important as they offer data on reproducibility while also giving clinicians the knowledge that multiple members of

the same class will yield similar results. There may be specific considerations (e.g., pharmacokinetics, off-target effects, cost, etc.), which clinically may drive the choice of a specific agent.

Early in the development of the cancer prevention program, it was decided to employ *in situ*-arising models of cancer (4) as contrasted with the use of either syngeneic cancer cell lines or xenografts. The rationale was that CADRg would be examining agents for their effects on cancer initiation and progression, as contrasted with therapeutic testing of fully progressed invasive cancers. Because the program was initiated more than two decades ago, many of the models initially employed were chemically induced. We have added several transgenic models as well (4). A brief description of the models is presented in Table 1, while a fuller description with certain references is in Supplementary Table S1. In brief, the two mammary cancer models are ER⁺ cancers induced by MNU in virgin female Sprague–Dawley rats, and a transgenic mouse model (MMTV-*Neu*/P53KO), which develops ER⁻ cancers overexpressing *Neu* and which have alterations in P53. This appears to be a reasonable model for *Neu*-overexpressing ER⁻ breast cancers in women as most of those tumors also have P53 mutations. The colon/intestinal tumor models are the AOM-induced rat model and the Min mouse. Tumors in these models have alterations in the Wnt pathway; AOM rat (β -catenin) and Min mouse (APC). Mutations in this pathway are associated with the preponderance of sporadic colon cancers and adenomas in humans (typically APC truncation mutations). The squamous cell skin model is induced by repeated UV exposure (similar to humans) and is driven by P53 mutations at dipyrimidine dimer sites; like humans. The last model is the OH-BBN-induced urinary bladder cancer model in rats. This model, although employing a synthetic organ-specific carcinogen, appears by gene array analysis to have significant overlap with invasive human bladder cancers (7).

Some characteristics of our screening are unique to this program and clearly differ from many smaller scientific laboratories. However, we will mention studies by other groups that demonstrate that they observed similar results. Our references to others is not an attempt to be all inclusive but rather to give by example relevant studies by authors other than ourselves. In fact many of the studies by other groups, which we quote, were agents that they initially tested and that we wished to confirm so that they might progress to clinical trials.

The CADRg functions via contract mechanisms, and fund either academic or contract laboratories to perform these studies; specifically, laboratories that have performed extensive studies with a specific animal cancer model. Thus, the laboratories employed are likely to have performed a given assay numerous times over an extended period. Furthermore, laboratories are employed that can determine the stability and concentration of agents in the

diet or in gavage vehicles. Finally, our initial screenings were performed with standardized protocols where administration of the agents was initiated shortly after the end of carcinogen treatment or in young transgenic animals, and continued throughout the duration of the study. Two additional generalized thoughts on our screening procedures warrant comment. An agent is considered effective if it achieves statistical significance in that model. For the MNU (ER⁺) model, the MMTV-*Neu* (ER⁻) model, the AOM colon model, the Min mouse, and the UV-induced squamous cell model, endpoints are based primarily on tumor multiplicity at the end of the study. Although for the two mammary models, palpation data allow one to determine tumor latency as well. For the urinary bladder cancer model, the primary endpoint is final tumor weights and the percentage of rats developing large (>200 mg) bladder cancers. Our maximal dosing for agents with clear human use is within 2-fold of the maximal standard human dose based on FDA scaling. Thus, for all the NSAIDs, dosing is at or below the human equivalent dose (HED), dosing for EGFR inhibitors again is close to the HED, SERMs are effective as preventive agents at or below the HED, and the aromatase inhibitors were effective again at or below the HED.

ER⁺ mammary cancers (Table 2 and 3)

MNU is administered to 50-day-old female Sprague–Dawley rats. The resulting cancers first appear within 6–8 weeks of MNU treatment, are ER⁺, and are similar to well-differentiated human ER⁺ breast cancers by array analysis (8). However, approximately 50% of these tumors have mutations in *Ha Ras*. Mutations in *Ha Ras* are not found in human breast cancers.

SERMs (Table 2)

The efficacy of tamoxifen in this model has been shown by other investigators, including Jordan and colleagues (9). Their work helped support some of the original clinical therapy and prevention studies with this agent. In 1994, we published results showing similarly striking results with tamoxifen and a second SERM toremifene (10): a 50% reduction in tumor multiplicity at 0.4–0.6 ppm, and greater than 90% efficacy at doses greater than 1.5 ppm with tamoxifen. Toremifene (5 ppm) reduced tumor multiplicity by roughly 60%. Finally, we have recently found that the SERMs bazedoxifene and arzoxifene were profoundly effective in this model. The SERMs examined were effective in the ER⁺ rat model at doses lower than their human equivalent dose (HED) based on FDA scaling factors; although one should be aware that the human doses were based on their use in therapy. We have also found that two aromatase inhibitors (vorozole and letrozole), both of which inhibit estrogen production, are profoundly effective. Vorozole is an agent we have used and published

Table 2. Efficacy of multiple agents of different classes in the MNU-induced model of mammary cancer in rats

Model	Class of agents	Specific agent	Dose	Tumor multiplicity decrease (%)	Reference
MNU Rat	SERM	Tamoxifen	3 ppm	>95	Unpublished
MNU Rat	SERM	Arzoxifene	1 ppm	90	Unpublished
MNU Rat	SERM	Toremifene	5 ppm	65	10
MNU Rat	SERM	Bazedoxifene	5 ppm	85	Unpublished
MNU Rat	Aromatase inhibitor	Vorzole	1 mg/kg BW/day	>95	11
MNU Rat	Aromatase inhibitor	Letrozole	1 ppm	>90	Unpublished
MNU Rat	RXR agonist	Targretin	>150 ppm	>80	22
MNU Rat	RXR agonist	UAB-30	200 ppm	66	23
MNU Rat	RXR agonist	4Me UAB-30	200 ppm	80	23
MNU Rat	EGFR inhibitor	Gefitinib	10 mg/kg BW	>85	16
MNU Rat	EGFR inhibitor	Erlotinib	6 mg/kg BW	>85	17
MNU Rat	EGFR inhibitor	Lapatinib	75 mg/kg BW	>85	18

* **Vorzole**: Agents in bold are effective as a therapeutic agent (reversal of palpable tumor) at a highly effective preventive dose. Most of the agents were not tested therapeutically. The SERMs required a markedly higher dose to achieve therapeutic efficacy than to achieve preventive efficacy.

repeatedly (11, 12), while we have tested letrozole more recently.

EGFR inhibitors (Table 2)

The EGFR inhibitors were among the first groups of targeted molecules developed for cancer therapy, primarily on the rationale that EGFR proteins were over expressed in the widest range of human tumors (lung, bladder, colon, head and neck, and breast; ref. 13). We will primarily discuss inhibitors of EGFR1. Increased expression of other EGFR proteins, particularly EGFR2 (Neu), is commonly observed in several tumors (particularly breast cancer) where overexpressed/amplified EGFR2 appears to be the driving alteration in roughly 20% of breast cancers. Determining the complete mechanistic effects of an EGFR1 inhibitor is complex due to the fact that EGFR molecules tend to function as dimers (often heterodimers), and modulation of EGFR1 may have secondary effects on heterodimers with EGFR2, EGFR3, and EGFR4. Interestingly, the luminal A (ER⁺/progesterone receptor [PR⁺]) breast cancers in humans tend to have relatively low expression of EGFR1. Nevertheless, there are at least two clinical studies (14, 15) showing that EGFR inhibitors appear to be substantially effective in the treatment of ER⁺/PR⁺ breast cancers. We mention this because the MNU rat model appears to be closest in appearance and

gene expression to well-differentiated ER⁺/PR⁺ human breast cancer. Our laboratory reported a decade ago that the EGFR inhibitor gefitinib was highly effective in both prevention and therapy in the MNU model (16). The dose of gefitinib employed in the therapeutic trial in the MNU model was the relatively effective preventive dose, implying that the therapeutic and preventive doses were quite similar. This finding that similar doses are required for prevention or therapy makes sense if one needs to achieve tissue levels above the K_i for EGFR1 phosphorylation for either activity. We confirmed these preventive and therapeutic results with another EGFR1 inhibitor erlotinib (17). The final agent examined as a member of this class was lapatinib, which was similarly effective in this model (18). Although this agent is listed as a combined EGFR1 and EGFR2 (Neu) inhibitor, it appears to be preferentially effective on EGFR2/Neu cancers. As stated above, however, altering EGFR2/Neu directly will have effects on heterodimers formed with other members of the EGFR family.

RXR agonists

A variety of agents that interact with the RXR receptors (RXR α , RXR β , and RXR γ) have been tested. Most of these agonists bind all three of the RXR receptors because all three have comparable binding sites. The standard

Table 3. Effects of multiple agents on development of ER⁺ cancers in the MNU-induced mammary model in Sprague Dawley rats; comparison of current and previous results

Agent (dose)	Tumor incidence ^a	Tumor multiplicity ^b	Tumor weight ^c	Reference ^d
Tamoxifen (2 ppm) ^a	100%* ↓/94%* ↓	100%* ↓/96%* ↓	100%* ↓/98%* ↓	10
Vorzole (1.2 mg/kg BW)	73%* ↓/74%* ↓	88%* ↓/90%* ↓	94%* ↓/ND	11
Bexarotene (Targretin) 150 ppm	77%* ↓/48%* ↓	93%* ↓/85%* ↓	95%* ↓/ND	22
Gefitinib (Iressa) 10 mg/kg BW	83%* ↓/78%* ↓	92%* ↓/80%* ↓	97%* ↓/98%* ↓	16
Atorvastatin (Lipitor) 200 ppm	5% ↑/0%	12% ↓/29% ↑	12% ↓/37% ↑	25
Metformin (150 mg/kg BW)	0%/0%	65%* ↑/23% ↑	63%* ↑/50%* ↑	27
Naproxen (400 ppm)	10% ↑/0%	40%* ↑/27% ↑	54%* ↑/38% ↑	28

^aFor example, Tamoxifen (2 ppm) tumor incidence: 100%* ↓/94%* ↓. Comparison is results for treatment group compared to a simultaneously performed control group. In a recent study tumor incidence was reduced 100%, while in the prior published results tumor incidence was reduced 94%. Final parameters (Incidence, multiplicity, tumor weight) at the end of the study are significantly different, $P < 0.05$; employing a non-parametric Wilcoxon Rank Test.

^bSimilar analysis for tumor multiplicity.

^cSimilar analysis for final tumor weights. Fifty percent with an arrow down (50% ↓) would be a 50% decrease in the parameter examined. Fifty percent with an arrow up (50% ↑) would be a 50% increase in the parameter examined. ND, Not determined in the prior study.

^dReference to prior published study examining the indicated agent.

retinoids (all *trans*-retinoic acid and 13-*cis*-retinoic acid), which bind and activate the retinoic acid receptors (RAR), do not bind the RXRs and have different toxicities and organ specificity than the RXR agonists. The RXRs form heterodimers with the widest range of nuclear receptors (PPAR α , PPAR γ , CAR, FXR, VDR, etc.), and alter transcription of the widest range of genes (e.g., ref. 19). Roughly 20 years ago, Gottardis and colleagues showed a synthetic RXR agonist, bexarotene, was highly effective in the prevention of MNU-induced ER⁺ tumors in the rat (20). They subsequently showed that bexarotene was similarly effective as a therapeutic agent in this model (21). We have since reproduced both the preventive and therapeutic findings with this agent (22). Furthermore, we and others have shown that a variety of additional RXR agonists are highly effective in the MNU rat model of breast cancer (23). The RXR agonists, because of their interaction with a wide variety of nuclear receptors, may have substantially different interactions with specific heterodimer partners, and may, therefore, induce different gene patterns (19). Nevertheless, multiple RXR agonists (e.g., bexarotene, LGD 100268, UAB-30, 4MeUAB-30) have all proven to be highly effective in the ER⁺ model of breast cancer (23, 24).

Negative results in the rat mammary model

As stated earlier, we have more limited data regarding agents that gave negative results. The reason several of these compounds were tested initially was a specific clinical interest in these agents (based on epidemiologic studies or published preclinical data), which encouraged their examination. The four agents/classes to be discussed are statins, metformin, NSAIDs, and ARE agonists (25–28). It was felt that negative results with these agents were likely to prove controversial, particularly with statins and metformin. Therefore, repeated studies were done with these agents before we published our initial results. The evaluations of NSAIDs were driven by the fact that these agents were highly effective in multiple cancer rodent models of various organs, (colon, urinary bladder, skin, and esophagus), and raised the possibility of a truly generalized preventive agent relevant to the widest range of organs. The antioxidant agonists (1,2 dithiol-3-thione, 5-MeCDDO) were examined because of profound scientific interest. An examination of Pub Med yielded greater than 1,000 publications in the last 10 years dealing with agents that activate the antioxidant response element (ARE). Recently, we directly repeated studies for a variety of positive and negative agents. These agents were tested in both standard diet (Teklad, 4% fat) and high-fat diet (42% fat) to compare the effects of different diets. However, the results in Table 3 compare the results using the standard Teklad diet in our recent studies with our published results with these agents that were collected over a period of up to 20 years. There is close agreement between the results in

our initial published studies and results in the second study; both for four positive agents (vorozole, bexarotene, gefitinib, and tamoxifen) as well as three negative agents (metformin, atorvastatin, and naproxen).

Preventive and therapeutic activity in the rat model (Table 2)

The ability of highly effective preventive doses to show therapeutic activity in this mode was also examined. Specifically, for a variety of the effective agents, it was found that they were, in fact, therapeutic in this model if the lesions were allowed to become palpable and only exposed to the agent at that time. These results were reported individually with a number of the highly effective agents, and addressed more systematically approximately 10 years ago in an article dealing with short-term efficacy biomarkers (28). We have reported that various agents, including aromatase inhibitors, RXR agonists, and EGFR inhibitors, are effective at similar doses in either a preventive or therapeutic setting (16, 17, 22).

ER⁻ mammary cancer (MMTV-Neu/P53KO) (Table 2)

This is a transgenic mouse with expression of EGFR2 (*Neu*) under the control of an MMTV promoter and the heterozygous knockout of P53 (29). The resulting tumors overexpress *Neu* and routinely have mutations in P53, which makes this an excellent model for roughly 20%–25% of human cancers that are driven by amplified *Neu*, and typically have P53 mutations. Our positive results in these studies reproduced the results of other investigators. Specifically, RXR agonists and EGFR inhibitors are highly active in this model (30, 31). We have replicated data with regards to bexarotene (RXR agonist; ref. 25) and EGFR inhibitors (gefitinib and erlotinib) (Oncology Reports, in press). It was further found that UAB-30, also a RXR agonist, showed significant efficacy in this specific model. The most surprising repetitions, given that the final tumors are ER⁻, are that the SERM (tamoxifen) exhibits significant activity when administered to mice beginning at a relatively young age. These data show that we can reproduce the strong positive results of others who employed the standard MMTV-*Neu* model; unlike ours, which has an additional modification in P53. Our laboratories have had many negative results in this model, including statins, NSAIDs, PPAR γ agonists (rosiglitazone), metformin (27), and 5MeCDDO (26). Results with metformin and celecoxib showed no activity, which was somewhat different from certain published reports that showed statistically significant results. One should be aware that the published studies, although positive, were strikingly less effective than the highly positive published results, we and others have obtained with EGFR inhibitors and RXR agonists (25, 30, 31). Our laboratories also observed the efficacy of both EGFR inhibitors and Targretin when administered to 100-day-old mice, who have preexisting preinvasive

lesions, whereas our standard protocol involves treatment of mice at 42–50 days of age.

Results in colon/intestinal models (Table 4)

The two models employed were the AOM colon model in rat, and the Min mouse model which primarily develops adenomas in the small intestine. One of the great appeals of modeling colonic/intestinal tumors is that there are driving genetic alterations associated with these tumors in both human and rodent. Specifically, mutations in the Wnt pathway, APC mutations in the Min mouse (32) and humans, and β -catenin mutations in the AOM rat. Both β -catenin or APC mutations result in increased levels of β -catenin, which binds to the transcription factors TCF and then activates the Wnt pathway.

AOM rat model (Table 4)

The AOM rat colon model was initially employed for testing preventive agents almost 30 years ago. It was shown that the NSAID piroxicam was highly effective in preventing AOM-induced tumors (5). The data were confirmed with piroxicam and with a wide variety of other NSAIDs, including sulindac, indomethacin, ibuprofen, flurbiprofen, and more recently, naproxen (discussed in ref. 33). Subsequently, we and others showed that several of the COX2-selective inhibitors (celecoxib, rofecoxib) were similarly highly effective in this model; supporting the hypothesis that inhibition of COX activity is a primary chemoprevention target. The coxibs are, in fact, no more effective than standard NSAIDs when tested at their HEDs (discussed in ref. 33). Various NSAIDs and celecoxib proved to be equally effective when administered after animals had developed ACFs or administered when early adenomas were present; implying that their effects were primarily on the progression stage of tumor development (5, 34, 35). All these agents were strongly effective at their human equivalent dose (HED), based on standard FDA scaling factors (33). Two additional agents of different classes have proven highly effective in this model: difluoromethylornithine

(DFMO; ref. 36), an ornithine decarboxylase inhibitor, and gefitinib, an EGFR1 inhibitor. Interestingly, DFMO has reproducibly shown activity in this model in conjunction with a suboptimal dose of an NSAID (36). These observations helped support an effective clinical trial combining sulindac and DFMO (37). The combination resulted in a 60% decrease in all adenomas, and a substantially greater effect on advanced adenomas (37), which are thought to be the precursor to human colon cancer. In contrast, a wide variety of agents have proven ineffective; including 1,2 dithiol-3-thione (an ARE agonist), metformin, 2-n-butylthione, calcium chloride, conjugated linoleic acid, dimethyl fumarate, fumaric acid, ellagic acid, enoxolone, EGCG, and genistein. An agent is considered ineffective if its efficacy in a given model fails to achieve statistical significance in that model.

Min mouse model (Table 4)

Almost 17 years ago, it was shown that the NSAIDs piroxicam (38) and sulindac (39, 40) were highly effective in preventing intestinal adenomas in the Min model. The data were confirmed with a wide variety of other NSAIDs, including indomethacin, ibuprofen, flurbiprofen, and more recently naproxen. Subsequently, we and others showed that a number of the COX-2-selective inhibitors (celecoxib and rofecoxib) were similarly highly effective in this model; supporting the hypothesis that inhibition of COX activity is a clear exploitable chemoprevention target (41).

Interestingly, these various NSAIDs and celecoxib are effective even though Min mice already have a considerable adenoma burden when treatment is initiated. All of these agents were highly effective at their HED, based on standard FDA scaling factors (33). The other agent/class of compounds that has proven highly effective in this model is the ornithine decarboxylase inhibitor DFMO. This agent has been reproducibly shown to be active in this model either alone or in conjunction with a suboptimal dose of an NSAID (42). These observations, together with those in the

Table 4. Efficacy of multiple agents of different classes in the AOM rat colon or min mouse models

Model	Class of agents	Specific agent	Dose	Tumor multiplicity decrease (%)	Reference
AOM Rat	NSAID	Piroxicam	150 ppm	>80	5
AOM Rat	NSAID	Naproxen	400 ppm	>80	Unpublished
AOM Rat	NSAID	Sulindac	200 ppm	>75	Unpublished
AOM Rat	NSAID	Ibuprofen	200 ppm	>75	Unpublished
AOM Rat	NSAID	Nimesulide	200 ppm	>75	Unpublished
AOM Rat	Coxib.	Celecoxib	1000 ppm	>85	35
AOM Rat	Coxib	Celecoxib	250 ppm	65	35
Min Mouse	NSAID	Piroxicam	150 ppm	>70	38
Min Mouse	NSAID	Naproxen	400 ppm	>80	Unpublished
Min Mouse	NSAID	Sulindac	200 ppm	>75	39, 40
Min Mouse	Coxib	Celecoxib	1000 ppm	>85	41
Min Mouse	Coxib	Celecoxib	250 ppm	65	41

^aAOM Rat: Agents in bold effective when administered beginning 12–14 weeks after AOM when aberrant crypt foci, but not clear adenomas, already exist. A few agents have been shown to inhibit progression from adenomas to adenocarcinomas but most agents have not been tested in such a protocol.

^bMin Mouse: All agents in bold effective in a 5–6-week-old Min Mouse which already has a substantial tumor (adenoma) burden. Thus, highly effective agents must be causing regression of some preexisting adenomas.

AOM-treated rat, helped support a strikingly effective trial by Meyskens and colleagues that combined sulindac and DFMO; mentioned above (37). In contrast, a wide variety of agents have proven ineffective, including 1,2 dithiol-3-thione (an ARE agonist), 5MeCDDO, bexarotene, fenretinide (structurally a retinoid that binds neither RAR or RXR receptors), and metformin.

UV-induced squamous cell cancer of the skin in SKH hairless mice (Table 5)

Our laboratories have also examined multiple agents in a model of squamous cell cancer (SCC) of the skin induced by UV light (43). The model is comparable to human SCC of the skin, exhibiting similar histopathology, is mediated by the same human carcinogen (UV light), and is similarly driven by p53 mutations due to formation of UV-induced dimers at dipyrimidine sites. Using the SKH hairless mouse was begun 17 years ago, and we have performed a variety of studies employing the UV-induced model. Initially, the COX-2 inhibitor celecoxib was shown to be effective, albeit no more effective than a standard NSAID (indomethacin) (43). Subsequently a variety of standard NSAIDs, including sulindac, celecoxib, indomethacin, naproxen, NO-naproxen were also found to be highly effective (44). Another agent that is highly effective is DFMO when administered alone or in combination with celecoxib by causing the regression of preexisting papillomas or even early squamous cell cancers (45, 46). Again, the number of negative or minimally effective agents is large; for example, vitamin E and PPAR γ agonists (47).

OH-BBN rat urinary bladder model (Table 5)

The last model to be discussed is one of invasive urinary bladder cancer induced by the organ-specific carcinogen OH-BBN. Unlike the models of colon/intestinal cancers, SCCs of the skin, and *Neu*-overexpressing breast cancer, there is no clear driving mutated gene

in human bladder cancer. Nevertheless, we have examined the rat bladder tumors and invasive human bladder tumors and shown substantial overlap based on gene expression (7). Interestingly, the agents that have proven particularly effective in the bladder model are effective in colon, specifically the NSAIDs/coxibs and EGFR inhibitors (6, 48). The initial observations of the efficacy of NSAIDs in rodent urinary bladder were observed almost 30 years ago (49).

Various studies initially showed that the COX-2 inhibitor celecoxib was effective; but again no more effective than other NSAIDs (33, 50). We also subsequently showed that sulindac, naproxen, and NO-naproxen were highly effective. More recently, our laboratories found that the various NSAIDs were effective in blocking progression to large, palpable invasive cancers in animals that already had microinvasive disease. However, they were not effective in blocking the development of hyperplasia or even microinvasive lesions (6); this showing that their primary effects were not on the earliest stages of tumor progression. It was similarly found that a variety of EGFR inhibitors (gefitinib, erlotinib, and lapatinib) were highly effective in this model, and (like the NSAIDs) were highly effective when microinvasive lesions already existed (6). Interestingly, the PPAR γ agonist rosiglitazone proved to greatly enhance tumor formation in this model over a wide range of doses and times of initiation (51). Our laboratories do not routinely examine tumor enhancement in our models because we are not performing a true standard carcinogenicity test, and are always looking at carcinogen-initiated animals. However, human cell lines with overexpression/amplification of PPAR γ proliferate rapidly in the presence of a PPAR γ agonist (52). This finding indirectly demonstrates the potential relevance of the OH-BBN-induced bladder cancer model. Again, the number of negative or minimally effective agents are multiple, including Targretin, 5-MeCDDO, DFMO, etc.

Table 5. Efficacy of multiple agents of different classes in the OH-BBN rat urinary bladder cancer model or the UV-induced model of squamous cell carcinomas in SKH mice

Model	Class of agents	Specific agent	Dose	Cancer multiplicity decrease (%)	References
OH-BBN Rat	NSAID	Naproxen	400 ppm	>80	6, 48
OH-BBN Rat	NSAID	No-Naproxen	560 ppm	>80	48
OH-BBN Rat	NSAID	Sulindac	400 ppm	>80	48
OH-BBN Rat	Coxib	Celecoxib	1000 ppm	>80	44-46
OH-BBN Rat	EGFR inhibitor	Gefitinib	10 mg/kg BW	>80	6
OH-BBN Rat	EGFR inhibitor	Lapatinib	75 mg/kg BW	>80	Unpublished
OH-BBN Rat	EGFR inhibitor	Erlotinib	6 mg/kg BW	>80	Unpublished
				Decrease in SCCs	
UV Mice	NSAID	Indomethacin	400 ppm	>80	44
UV Mice	NSAID	No-naproxen	560 ppm	>80	43
UV Mice	NSAID	Sulindac	150 ppm	>80	43
UV-Mice	NSAID	Naproxen	400 PPM	>80%	43
UV Mice	NSAID	Aspirin	560 ppm	>80	43
UV Mice	Coxib	Celecoxib	1000 ppm	>80	43, 44

^aOH-BBN Rat: Agents in bold also effective when administered 14 weeks after last OH-BBN when roughly 50% of rats have microcarcinomas.

^bUV Mice: Agent in bold effective in UV exposed mice when they already have substantial papilloma and SCC burden. Thus, celecoxib, for example, could cause regression of lesions.

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Observations and Conclusions

Direct reproducibility (testing the same agent repeatedly; Table 3)

The data reported show that in various *in situ*-arising animal cancer models, the chemopreventive results obtained over a period of many years are quite reproducible. This conclusion is based primarily on results for highly effective agents, which are the primary agents that were repeated. We have tested a more limited number of negative agents, primarily in the MNU model (e.g., metformin, Lipitor, and various NSAIDs), and have demonstrated the reproducibility of the results with these specific agents.

Reproducibility as determined by multiple agents in the chemical/mechanistic class (Tables 2, 4, 5; Supplementary Fig. S1)

Perhaps the most important observation regarding reproducibility is that multiple agents of a given class give the same result. This has the advantage that if there are specific characteristics of a given agent that appear advantageous (e.g., dosing, toxicity or cost), the agent is likely to be as effective as most other members of the same class. These results also argue indirectly that for most of the highly effective classes of agents (COX inhibitors, SERMs, EGFR inhibitors) the off-target effects are unlikely to be driving efficacy. Thus, if one hypothesizes that off-target effects of a given agent are likely to drive its efficacy, then achieving similar efficacy in each organ with multiple agents of the same class would appear surprising. Particularly, as many of these agents are structurally varied (Supplementary Fig. S1). We have discussed this point specifically regarding the COX inhibitors (33).

There is one further appeal of the finding that agents of a given class work similarly. Biomarkers relevant to one member of the class are likely to be relevant to other members of the same class. As might be expected, all EGFR inhibitors (gefitinib, erlotinib, and lapatinib) decrease the phosphorylation of EGFR, ERK, and AKT. More striking, we found that RNA expression alterations by the aromatase inhibitor vorozole in palpable lesions in the MNU mammary cancer model had substantial overlap, at both the pathway and specific gene levels, with alterations seen in women treated with the aromatase inhibitors letrozole and anastrozole in presurgical clinical breast cancer trials (53). Thus, the RNA changes were similar across species, and were observed with different inhibitors of the same class. Therefore, biomarker changes observed with one member of the class are likely to be relevant to other agents in the same class.

Late interventions as an indicator of reproducibility

As discussed earlier, it is felt that a late intervention in the tumor progression process is more indicative of how phase III clinical prevention trials will be performed.

These have typically employed a tumor endpoint after five years of treatment. In the MNU mammary cancer models, we have shown that a wide variety of agents including aromatase inhibitors, SERMs, RXR agonists, and EGFR inhibitors all can be therapeutic in this model. In the colon models, it was shown that NSAIDs and DFMO are still highly effective when administered after ACFs already existed in the AOM-induced rat model and even in Min mice where all interventions are initiated in the presence of a significant tumor burden. In the OH-BBN bladder model, agents are still effective when administered to rats that already have preexisting microscopic transitional cell cancers. Finally, it was shown that celecoxib and DFMO given alone, but particularly when administered simultaneously, were highly effective in regressing or slowing the growth of existing tumors in the UV model of squamous cell skin cancer. We must, nevertheless, reiterate the fact that (in general) the animal tumors are not as advanced in terms of genomic alterations as are human tumors.

Reproducibility of suboptimal doses

Most of the repeat studies have involved highly positive agents. The only repeat tests with moderately effective agents are suboptimal doses of highly effective compounds based on the premise that these lower doses might be used in combination with a second agent to achieve preventive activity and potentially reduce toxicity. The rationale for such a combined approach is twofold: (i) the two different agents will preferentially affect different targets and may show synergy in terms of efficacy and (ii) because the toxicities of the two agents are likely to be different in the combination, the level of toxicity associated with higher levels of either specific agent might be reduced. Typically, we have attempted to use levels of each agent that achieve 40%–50% efficacy by themselves. In certain of these repeats, we have used relatively minor variations in dosing ($\leq 50\%$). However, when giving a suboptimal dose of an effective agent that achieves 45%–65% efficacy, one would presumably obtain statistical significance. In contrast, 30%–40% efficacy that reflects clear pharmacologic and physiologic effects may not achieve statistical significance. This is clearly not a reflection that one time the agent "worked" and one time it did "not work" because at slightly higher doses, the agent is invariably positive.

We have repeatedly tested three agents at suboptimal doses in the mammary cancer model (Supplementary Table S2): the aromatase inhibitor vorozole (0.12–0.16 mg/Kg BW/day, i.g.); tamoxifen (0.4–0.6 mg/Kg diet), and the EGFR inhibitor gefitinib (2–3 mg/Kg BW/day, i.g.). The tumor multiplicity of MNU treated control rats varied from a low of 3.9 cancers per rat to a high of 7.4 cancers per rat in the eight studies presented. In four studies with MNU-treated rats, the efficacy of tamoxifen [ratio of tumors (tamoxifen treated/vehicle

treated)] varied in the reduction in tumor multiplicity by 31%–51%. The 31% reduction has a $P > 0.05$, while all the others achieved statistical significance. In MNU-treated rats, vorozole (0.12–0.16 mg/Kg BW/day) showed efficacy; vorozole-treated/vehicle-treated varied in tumor multiplicity reduction by 35%–47%. The 35% reduction was not statistically significant, whereas it was in the other two studies. The EGFR inhibitor gefitinib similarly yielded reproducible results in the MNU model; showing 50% and 54% reductions in tumor multiplicity in the two studies, respectively.

The other model for which we have repeat data at doses of moderate activity is the urinary bladder model in which a moderately effective dose of the NSAID naproxen (30–40 mg/Kg BW/day) was used repeatedly. In three studies, the incidence of large tumors (>200 mg) was reduced 60%–65% and the effects on final cancer weights was reduced by 40%–48%. The effects on the incidence of large tumors and tumor weights were statistically significant in all three studies.

Clinical studies that relate to agents tested

Finally, for breast, colon, and skin cancers, there are positive clinical prevention data that can be compared. Although that is not the primary objective of this article, it is an important correlate. There are large clinical trials with the SERMs (54) and aromatase inhibitors (55) in human breast cancer that demonstrated their efficacy. There are also human colon adenoma trials demonstrating the efficacy of NSAIDs and coxibs (discussed in ref. 33), as well as the combination of the NSAID sulindac and the ornithine decarboxylase inhibitor DFMO (37). There are also recent clinical data in persons with familial adenomatous polyposis that the combination of sulindac and the EGFR inhibitor erlotinib is highly effective. Each of the individ-

ual agents were positive preclinically. Finally, there are data with celecoxib administered orally (56) and the NSAID diclofenac administered topically (57) that demonstrate the efficacy of COX inhibitors in the prevention of progression to SCCs of the skin.

Reproducibility of results and conclusions

As discussed in the Introduction, there have been questions raised regarding the reproducibility of portions of the scientific literature (e.g., 1, 2). There has been an effort to reproduce the findings of 50 articles published in high impact journals (3). Most of the studies involved have reflected important mechanistic questions and the results of the reproducibility studies have yielded "muddy results" (3). The types of mechanistic studies, often performed in cell culture, examined in "The Reproducibility Project: Cancer Biology" are quite distinct from the studies presented in this review. The studies presented involve *in vivo* screening efforts using standardized protocols in models that our contractors and others have employed numerous times. Furthermore, as stated above, we have primarily examined agents that yielded strong positive results. It is indeed the primary objective of our screening to identify agents that are highly effective in standardized preclinical models that might progress to the more heterogeneous venue of clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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