Published in final edited form as:

Free Radic Biol Med. 2011 September 1; 51(5): 931–941. doi:10.1016/j.freeradbiomed.2011.04.014.

Translational Research Involving Oxidative Stress and Diseases of Aging

Robert A. Floyd^{1,2}, Rheal A. Towner^{3,4}, Ting He³, Kenneth Hensley^{5,6}, and Kirk R. Maples⁷

¹ Experimental Therapeutics, Oklahoma Medical research Foundation, Oklahoma City, OK

² Dept of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK

³ Advanced Magnetic Resonance Center, Oklahoma Medical Research Foundation, Oklahoma City, OK

⁴ Dept of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK

⁵ Dept of Pathology, University of Toledo Medical Center, Toledo, OH

⁶ Dept of Neurosciences, University of Toledo Medical Center, Toledo, OH

⁷ Anacor Pharmaceuticals Inc, Palo Alto, CA USA

Abstract

There is ample mounting evidence that reactive oxidant species are exacerbated in inflammatory processes, many pathological conditions and underlying processes of chronic age-related diseases. Therefore there is increased expectation that therapeutics can be developed which act in some fashion to suppress reactive oxidant species and ameliorate the condition. This has turned out to be more difficult than at first expected. Developing therapeutics for indications where reactive oxidant species is an important consideration presents some unique challenges. We discuss important questions including whether reactive oxidant species should be a therapeutic target and the need to recognize the fact that an antioxidant in a defined chemical system may be a poor antioxidant operationally in a biological system and the importance of considering the fact that reactive oxidant species may accompany the disease or pathological system rather than being a causative factor. We also discuss the value of having preclinical models to determine if the processes which are important in causing the disease under study is critically dependent on reactive oxidant species events and if the therapeutic under consideration quells these processes. In addition we discuss measures of success that must be met in commercial research and development in preclinical and clinical trials and discuss as examples our translational research effort in developing nitrones for the treatment of acute ischemic stroke and as anti-cancer agents.

Introduction

It has been clear for many years that reactive oxidant species are strongly associated with many pathological conditions and disease processes especially those associated with aging. The earliest clear-cut evidence of an association was the recognition that ionizing radiation caused the formation of oxygen free radicals and also caused tissue injury and then later on

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cancer. The association of free radicals with ionizing radiation made it possible to draw a strong parallel association of oxygen toxicity to ionizing radiation damage [1]. This began slowly at first postulates that free radicals played a role in aging [2] and cancer development [3] followed by ever increasing accumulating reports on the importance of reactive oxygen species (ROS) in biological systems. The landmark discoveries of superoxide dismutase [4] and more rigorous methods of detecting, characterizing and quantifying ROS as well as biochemical reaction products formed by reacting with ROS [5, 6] lead to an ever increasing number of high quality reports. The realization that oxidative stress was a natural consequence of all biological systems dependent upon oxygen for life [7, 8] and that ROS were used as normal signaling agents and not just agents that caused damage lead to a plethora of reports that gave birth to our current knowledge of the multifaceted action of reactive oxidant species in normal biological processes, in pathological conditions, and in many disease processes.

Increasing numbers of observations have clearly demonstrated that reactive oxidant species are involved in the fundamental mechanisms that occur in the development of cancer, stroke, Alzheimer's disease, Parkinson's disease, cardiovascular disease, arthritis, diabetes, and many other diseases of aging as well as in many pathological conditions. This led to increasing optimism that novel therapeutics can be developed to help abate these diseases by acting to mitigate against the action of reactive oxidant species in the development of these diseases. This has turned out to be not as simple and straight forward as it would appear. This report has as one of its goals to critically delve into various important concepts, milestones, and caveats involved in translational research in this area and to also summarize, as examples, some of our translational research and development effort in this area.

Important Concepts in Translational Research and Reactive Oxidant Species

Biological Reactive Oxidant Species

It is now widely recognized that biological systems that require oxygen for life are under various levels of oxidative stress at all times. Biological systems produce various reactive oxidant species, which we will term ROxS, during normal conditions and in disease states and when they are under various pathological conditions. The reactive oxygen species produced include superoxide, hydrogen peroxide, hydroxyl free radicals, as well as singlet oxygen in some conditions. In addition nitric oxide is produced. Nitric oxide can undergo numerous possible reactions with biological molecules to produce various reaction products that have important consequences in biological processes. For instance nitric oxide can react with superoxide to produce peroxynitrite that can readily react with biological molecules. Peroxynitrite can react with carbon dioxide which is considered an inactivation reaction. In this report we are defining reactive oxidant species as referring to reactive oxygen species plus reactive nitric oxide species.

Should Reactive Oxidant Species be a Therapeutic Target?

Successful translational research depends upon many factors including accurately defining and thoroughly characterizing the therapeutic target. This becomes very important and complex when the translational research is conducted for indications where ROxS are considered to be an important factor in the disease or pathologic state. Many times in the past ROxS per se have been tacitly considered the therapeutic target and this, it seems, is a mistake to do so. A few examples are illustrated below. The therapeutic target may involve various unknown factors that may cause a sustained enhanced level of ROxS which characterizes the disease or pathologic state; for example. ROxS by their nature are reactive and therefore not at a constant concentration in contrast to other cellular constituents, such

as specific proteins that are generally fairly long lived and relatively stable and are considered therapeutic targets in various conditions. ROxS at very high levels are damaging to the cell but at moderate to low levels can be considered as agents that the cell may use to conduct various aspects of the necessary processes that cells conduct such as growing, reacting to stress, changing function as in a pathological state, or even dying. Therefore it is important to conceptually realize that ROxS are really not usually the actual therapeutic target per se but rather they are acting perhaps uniquely upon biological molecules carrying our processes that turn out to be important steps in the disease and/or pathological process and as such this may turn out to be the therapeutic target. Therefore it is extremely important to very thoroughly characterize the mechanistic basis of how ROxS acts in the specific steps of the disease and/or target indication of the translational research effort. This is the reason that most of the early phase preclinical research and development turns out to be directed toward perfecting animal models and to accurately characterizing and defining the mechanistic basis of the therapeutic target.

Perhaps the first attempts to treat or modify the rate of aging using antioxidants was done in a series of experiments [9–12] by Denham Harman, the pioneer who first began to espouse the “free radical theory of aging”. These experiments, conducted in mice over the 1969–1980 time interval, were based on the premise that free radical reactions contribute significantly to the degradation of biological systems and that this was important in aging and therefore that administering dietary antioxidants would increase life-span. Thus biological free radicals were considered the therapeutic target and aging was the indication and that well known chemical antioxidants including, among others, butylated hydroxy-toluene, santonin, and vitamin E were proposed to mitigate the free radical processes and thus increase life-span. The experimental design and protocol were logical in the light of the current knowledge at that time. However the experiments in general showed at best only a very modest if any effect with one or more of the antioxidants in some cases but not in a consistent manner as would be expected if the idea being tested was as straight forward as expected as, for example, that which would occur in the use of the antioxidants in the carefully defined and well characterized chemical systems [13–15]. It should also be noted that in the light of current practices of rigorous animal husbandry, choice of animal species lacking pathological problems as well as careful monitoring of dietary food intake and accurate monitoring of antioxidant bioavailability, the results obtained were at best questionable. These experiments are highlighted for historical reasons as well as for the fact that they represent the first of many mostly unsuccessful attempts since then to directly extrapolate chemical reaction-based knowledge about antioxidants and biological free radicals into practical biomedical applications. Nevertheless important knowledge were garnered from these bona fide and heroic efforts as well as many other very carefully done studies, some conducted recently [16, 17], to evaluate if antioxidants do increase lifespan. It is now prudent to reflect and try to understand the lessons learned from these experiments. A recent critical perspective mini-review by Gutteridge and Halliwell [18] notes the problems associated with over extrapolation of antioxidants as “elixirs of youth”.

Is there Confusion about the Meaning of the term Antioxidant?

Antioxidant is a term widely used in the oxidative stress literature as well as in translational research literature but there appears to be much confusion about its actual meaning as used in translational research applications. The term antioxidant was traditionally defined by Gutteridge and Halliwell as “any substance that delays, prevents or removes oxidative damage to a target molecule” and is still widely used [19] however they recently noted that it can be defined in multiple ways depending on the methods used to measure antioxidant activity [18]. The confusion in translational research seems to arise from the use of the term antioxidant as defined in the strict chemical reaction-based definition for a chemical which

is then used in biological systems where the operational use of the term antioxidant is much more meaningful. For example there are numerous instances where a chemical that is a very potent antioxidant in defined chemical systems is used in biological systems, where known reactive oxidant species are involved, but is found to have no activity at all. On the other hand, there are numerous instances where specific chemicals that are poor antioxidants in well defined chemical reaction systems may in contrast act as very potent agents in quelling biological processes where biological free radical processes are involved. One specific example of this is the PBN-nitrones. It should be noted that PBN refers to α -phenyl-*tert*-butylnitron. In general the PBN-nitrones are only modest antioxidants in most lipid peroxidation reactions [20–25] but have very powerful biological effects in several disease models that are known to involve ROxS processes [26].

Winterbourn provided a rigorous review [18] of endogenous reactive oxidant species and their endogenous targets and the limitations of added radical scavengers. Based upon reaction rates of each specific ROxS with known physiologically relevant targets she noted that the diffusion distance of specific cellular oxidants is different depending upon the cellular compartment where it is formed and if it is enriched in reactant targets or not [19]. For example she noted that the H_2O_2 diffusion distance is decreased a thousand-fold, ie from 1.5mm to approximately 1.5 μm , depending upon the presence of biologically relevant levels of peroxiredoxin 2. In the same vein Jones also has emphasized the importance of redox compartmentalization in cellular oxidative stress [27] as well as the importance of the thiol proteome in cellular response to H_2O_2 [28]. The reaction rate of hydroxyl free radicals is about $10^{10} \text{ M}^{-1}\text{s}^{-1}$ (essentially diffusion limited) and they react indiscriminately with almost any biological molecule encountered. This means that exogenously added radical scavengers would have to be present at unrealistically high concentrations to be able to compete with endogenous targets [19]. Along with their limited bioavailability, this is likely the reason why many excellent “antioxidants” in chemical systems prove to be ineffective in experimental biological models.

PBN-Nitrones and Signal Transduction Processes

It is important to point out that despite the fact that the PBN-nitrones have powerful biological effects in several experimental models it is highly unlikely that the mechanistic basis of their activity is not due to their spin-trapping activity but due to their ability to suppress exacerbated cell signaling processes and to act as powerful anti-inflammatory compounds [26, 29, 30]. It is likely that the mechanistic basis of the biological activity may differ between PBN and some of its potent derivatives such as 4-hydroxy-phenyl-*tert*-butylnitron and 2,4-disulfonyl-phenyl-*tert*-butylnitron but it is also likely that some mechanistic commonality is shared by these nitrones also. A systematic study of their effects on inhibiting inflammatory processes and/or signal transduction processes comparing PBN versus its various potent derivatives has never been done. Tissue specific enhanced cellular signal transduction processes are hall marks of inflammatory processes and anti-inflammatory agents in general act to quell these processes. Many studies involving animal models do suggest that PBN exhibits effects that strongly implicate anti-inflammatory activity [29–32]. Cellular signal transduction studies involving LPS-mediated activation of primary macrophages showed that induction of inducible nitric oxide synthase (iNOS) as well as COX2 mRNA and protein in these cells were both suppressed by PBN but the amount of PBN required was in the 1–5 mM range [33]. It was shown that PBN did not catalytically inhibit the iNOS enzyme per se and had only limited effect on COX2 activity at supra-pharmacological levels [33]. One study that provided an important suggestion regarding mechanism of action of PBN in signal transduction involved the use of primary astrocytes which were activated by IL1 β , H_2O_2 or hyperosmolarity [32]. In this study we found that activation of MAP-kinase P₃₈ was prevented by pretreatment with 1mM PMN or

NAC (N-acetyl-cysteine). In this study we also showed that secondary production of H_2O_2 was evoked by activating the cells with $\text{IL1}\beta$ or hyperosmolarity. Evidence was also presented which strongly implicated that the H_2O_2 produced mediated signal transduction by transiently inactivating a phosphatase [32]. Only limited studies have been done using 2,4-disulfonyl-phenyl-*tert*-butylnitron where parameters that relate to inflammatory processes were assessed. It was shown that iNOS expression in glioma was suppressed [34]; and in an experimental stroke model cytochrome c release was suppressed by this compound [35].

Therapeutic Targets in Neuroinflammation: Pharmacological vs. Chemical Antioxidants

It is now widely accepted that many clinically distinct neuropathological conditions of aging, including common diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) but also less common conditions such as amyotrophic lateral sclerosis (ALS), all involve a major component of neuroinflammation [36–39]. Neuroinflammation is an innate immune response of the central nervous system (CNS) that entails pathological activation of astrocytes and microglia, the brain-resident macrophage [36, 37, 40]. One of the most prominent features of reactive glia is the production of ROxS including H_2O_2 and •NO-derived species, largely through cytokine-mediated recruitment of NADPH oxidase and induction of iNOS expression [37, 38]. These ROxS can directly damage neurons or indirectly perturb the neuronal environment by stimulating redox-sensitive signal transduction processes linked to cytokine and prostaglandin production, thus driving a positive-feedback cycle of neuroinflammatory progression. Thus, ROxS arguably are targets for therapeutic intervention in a variety of neuropathological conditions. The brain poses a special problem for neurotherapy efforts. However the blood-brain barrier (BBB) prevents or inhibits passage of xenobiotics from the blood stream into the brain parenchyma, thus limiting the ability of classical antioxidants from reaching tissue concentrations that could be expected to provide relief from excess ROxS through purely chemical mechanisms such as radical chain-breaking or antioxidant enzyme mimicry.

A more feasible approach for mitigating brain ROxS is through pharmacological antagonism of the enzymes and systems responsible for the ROxS production. Such approach would require only that small concentrations of drug penetrate the BBB, such that pharmacologically active concentrations are achieved near the site of ROxS production. It has been argued that many of the neuroprotective actions in animal models that are ascribed to natural botanical antioxidants, such as flavanoids, polyphenols, and tocopherols, actually derive from the pharmacological antagonism by these compounds of ROxS-producing enzyme systems [39, 41]. As a case in point, SOD1 mutant mouse models of familial ALS are well-established to suffer extreme protein oxidation as part of a robust neuroinflammatory phenotype that appears largely driven by pro-inflammatory cytokines of which tumor necrosis alpha (TNF α) is a principle agent [37, 38, 42–46]. Chemical antioxidants, such as metalloporphyrins that chemically mimic SOD1 by catalyzing superoxide disproportionation, have met with preclinical success in slowing ALS-like disease in the rodent [47] which thereby largely validates the oxidative stress component of ALS pathogenesis at least in murine models. However, drugs need not be direct radical scavengers to reduce ROxS. Small molecule inhibitors of TNF α signaling or production have shown admirable protective efficacy in the SOD1^{G93A} mouse model of ALS [45, 46, 48], though adverse events or patient intolerance have so far prevented application to human clinical disease. Agents that block or antagonize TNF α in microglial cell culture can potently suppress iNOS expression and reactive nitrogen species generation [37, 38], thus acting functionally as antioxidants, without the need to invoke direct radical-scavenging action of the drugs in question. Conversely, scavenging ROxS with SOD1 mimics has been found to attenuate inflammation in stroke models [49], demonstrating that ROxS and

neuroinflammation are intimately entwined in some cyclical fashion that is difficult to empirically separate within animal models of neurodegeneration or neurotrauma.

In some cases both the chemical and pharmacological antioxidant actions of certain natural products may map to the same physical-chemical features of the molecule. For example the natural product nordihydroguaiaretic acid (NDGA), which inhibits TNF α activation of microglia probably through a mechanism involving NDGA inhibition of arachidonate 5-lipoxygenase (5LOX), is an excellent chain-breaking antioxidant that once was used as a food preservative [44]. The unique tethered dicatechol structure of NDGA imbues it not only with the ability for form stabilized phenolic radicals, but also allows NDGA to trap the iron center of 5LOX in the reduced ferrous state, while sterically blocking the 5LOX active site [50]. When 5LOX is pharmacologically removed from the network of TNF α -stimulated microglial pathways, the biological response of the cell is significantly attenuated, particularly with respect to RNS production [44]. Thus the same physical-chemical properties that often impart chemical antioxidant potential to a particular pharmacophore, also allow the molecule to exert leverage as a drug through blocking pathways of ROxS production. Interestingly, chronic oral administration of NDGA provides one of the few examples wherein a natural product has been shown to increase lifespan of outbred mice [17], suggesting that either the pharmacological or chemical properties of this molecule may have greater implications to the biology of normal aging. Structurally similar polyphenols have been documented to extend yeast replicative lifespan through a mechanism of lowering K_m of sirtuin family NAD⁺-dependent protein deacetylases [51]. We are not aware of studies that have investigated NDGA for sirtuin activating potential, however this possibility is plausible and worthy of future consideration.

Gamma-tocopherol and neurodegenerative diseases. Despite the above-mentioned limitations of the BBB in preventing CNS accumulation of xenobiotic antioxidants, there are specific cases where natural micro-nutrients with specific chemical (chain-breaking) and pharmacological antioxidant potential do accumulate in the aging human brain so as to reach appreciable levels. Alpha tocopherol (vitamin E) and the less-methylated gamma tocopherol (γ -T) are important cases in point that deserve particular mention. The literature debating whether vitamin E is neuroprotective against age-related brain diseases, including AD and PD, is voluminous and contentious and reviewed elsewhere [39]. Gamma tocopherol is less well studied but accumulates to levels similar to α -T in AD brain, and is prone to nitration through reaction with RNS [52, 53]. The product 5-nitro-gamma tocopherol (5NO₂- γ -T) is elevated in AD brain in the same regions that protein nitration has been found elevated [52], and is more potent than α -T at protecting isolated brain mitochondria from damage inflicted by the peroxynitrite generating compound SIN-1 [52]. Because α -T does not undergo irreversible head-group nitration in the same fashion as γ -T, γ -T may uniquely scavenge RNS in the lipid milieu of the aging brain. Furthermore, γ -T reportedly antagonizes prostaglandin E₂ (PGE₂) synthesis through the cyclooxygenase pathway by a pharmacological mechanism not shared with α -T [53–55]. These findings raise the interesting possibility that this natural botanical antioxidant and human micronutrient may indirectly affect the neuroinflammatory potential of aging brain.

Nitrones; their action in preventing neuroinflammation. The PBN-nitrones provide an excellent example of an agent known to trap free radicals and as such thus considered an antioxidant yet the mechanistic basis of its potent activity in suppressing neuroinflammatory processes is not due to its direct chemical ‘antioxidant-type’ activity but due to its anti-inflammatory activity. A clear-cut demonstration of the ability of PBN to suppress neuroinflammatory processes is provided in two animal models; A) the rat kainic acid (KA) induced epileptic-type seizures [56] and B) the rat LPS-brain (intracisternal) injection model of neuroinflammation [57]. In the KA model PBN was administered (IP) at the same time as

KA. PBN significantly suppressed seizure intensity at 30 min and completely prevented mortality at 4 days and nearly completely suppressed KA-induced hippocampal signal transduction signaling in the AP-1, NF κ B and P38 MAP kinase pathways and prevented cellular killing and prevented cellular apoptosis at 4 days as revealed by TUNEL staining. The hippocampal cell killing was prevented if PBN was given even 30 min after KA injection. In the LPS brain injection model PBN was administered (~60 mg/kg/day) in drinking water starting 24hrs after LPS administration and continued for 6 days when brain inflammation was assessed by staining cryostat sections with [³HPK1195] as a marker of activated microglia and [¹²⁵I]iodoMK801 as a marker of open-channel activated state of NMDA receptors. LPS increased microglia activation by 2–3 fold in the temporal and entorhinal cortex, hippocampus and substantia innominata areas and this was accompanied by a significant (>50%) decrease in NMDA receptors in the same areas. PBN administration caused an ~25% decrease in microglia activation and a complete reversal of the LPS-mediated decrease in NMDA receptors. Loss in NMDA receptors is thought to contribute to the decrease in cognitive function in diseases associated with neuroinflammation such as Alzheimer's Disease.

Antioxidants and Cancer Therapeutics

There is significant research activity in the potential use of antioxidants as cancer therapeutics as well as the use of antioxidants as complimentary additions to suppress the toxic side effects of standard cancer chemotherapeutics and radiation treatments. Several reviews in these research areas have been published recently. Complimentary supplements, some of which are antioxidants, are currently being used along with standard cancer therapy in many countries now [58]. For example selenium supplements to enhance standard chemotherapy and radiotherapy is being used. There appears to be a significant need to address the cardiotoxicity that accompany the use of many standard cancer therapeutics [59]. The well known cardiotoxic side effects of adriamycin, considered to be due to enhanced oxidative damage to the heart, serves as a classic example. Antioxidant supplements have been widely used by patients that have undergone standard breast cancer treatment; however it appears to be too early to draw general conclusions as to its effectiveness [60]. Melatonin has potent antioxidant properties and its use as a supplement to standard breast cancer therapy seems to have significant promise [61, 62]. Resveratrol has been shown to suppress all stages of cancer development in experimental models and is now in early stages of clinical trials [63]. Preclinical as well as clinical trials are ongoing regarding the use of green tea polyphenols for treatment of prostate cancer [64].

Measures of Success

Patented Discoveries and Decisions to Commercialize. In the course of biomedical research occasionally a finding occurs which can be of consequential significance to merit it being patented as a discovery. Since this entails significant cost much deliberation usually occurs as to whether its merits patenting, which may take several years. If the decision is made to seek patenting the discovery it will usually require additional research to explore the breadth and extent to which it can be extended in the biomedical arena. If further research in both the experimental as well as the scientific literature continues to add to the importance of the discovery then deliberations will usually be made as to whether to pursue commercial development of the technology. If decisions are made to pursue this route it is important to realize that this usually will require utilizing other types of expertise to continue down this path. In the course of commercial research and development there are measures of success that must be met. They are briefly discussed in the following sections.

Defining Preclinical Endpoints

There are many aspects to preclinical endpoints that need to be kept in mind during the development process. Preclinical endpoints need to include not only efficacy measures, but also evaluations of the toxicity of the compounds, their pharmacokinetics, the extent of their drug metabolism, and the establishment of chemical manufacturing processes and controls.

In order to evaluate a new therapeutic in human clinical trials in the US, the FDA requires the submission and FDA review of an Investigational New Drug (IND) application. This IND application includes proscribed sections covering the primary pharmacology (efficacy), secondary pharmacology (cardiovascular, respiratory, and CNS effects), pharmacokinetics and drug metabolism, toxicology (systemic toxicity in both rodent and non-rodent species), chemical manufacturing and control, and the proposed clinical trial protocol. These requirements are delineated in Part 312, Investigational New Drug Application, Code of Federal Regulations, Title 21 [65]. The studies for each of these sections are compiled into summary documents, which are then further summarized into the Investigator's Brochure. The IND is reviewed by FDA experts in each area, and then all of the results are evaluated in total. Investigator's need to demonstrate to the FDA that the proposed human clinical trial has been designed in such a way to insure the safety of the subjects involved. This includes demonstrating that there is a sufficient safety margin between the exposure that is predicted to be needed for efficacy and the exposure for which animal toxicities were found. In addition, the investigators must demonstrate that the drug product can be manufactured under Good Manufacturing Practice (GMP) guidelines and that it will be stable during the duration of the proposed trial.

The preclinical studies need to be matched to the expected clinical trial design. It will be required to conduct toxicology studies in rodent and non-rodents for a duration at least as long as your planned clinical trial. Table 1 lists the standard required studies and their associated costs for a small molecule new chemical entity (NCE) intended for oral treatment if the proposed use is 14 days or less. These studies will cost approximately \$1.5M for a NCE that has a straight-forward synthetic pathway. Exact study costs will vary depending on the choice of available contract research organization (CRO) and contract manufacturing organization (CMO), on the final exact study designs, and on the complexity of the manufacture of the active pharmaceutical ingredient (API) and the drug product. In addition, if unexpected adverse effects are found in these studies, additional work may be required. The safety and toxicology studies will require analysis of the dosing solutions and toxicokinetic analyses to evaluate plasma drug exposure during those studies. These costs are reflected under Pharmacokinetics and Drug Metabolism. This set of studies will take about 6–9 months to complete if conducted in parallel whenever possible. Development begins with the synthesis of the API followed by the drug product. Once the API is available, the toxicology studies are on the critical path. The rest of the studies can typically be completed if run in parallel with the 14-day toxicity studies. Thus, critical path is typically manufacture of the API followed by the completion of the 14-day toxicity studies. It is necessary to have at least audited draft reports for all studies at the time of submission of the IND.

Drug development is most often based on the concept that exposure equals result. In order for a drug to work in people, a sufficient dose must be given to reach a desired efficacious concentration. Likewise, toxicity measures are also linked to exposure results. Preclinical studies are therefore conducted to establish what exposures are needed to achieve efficacy and at what points will exposure lead to potential toxic effects. The toxicology studies are required to be done in both rodent and non-rodent species in order to evaluate if the exposures required for toxicity are similar in different animals. The results of the pharmacokinetic studies in different species allows investigators to use intraspecies scaling

methods to predict what dose would be required in human clinical studies to achieve the desired efficacious exposure.

In a perfect setting, the preclinical efficacy model will be one that has already been validated through both animal model and human clinical testing. This validation infers that the activity seen in the animal model was proven to be predictive of what was found in man. Quite often, however, the proposed mechanism of action of the compounds in development are sufficiently novel that validated animal models do not exist. In the absence of a validated model, researchers must choose the model they feel best mimics the disease of interest. This is most often an animal model, but *in vitro* efficacy models may also be used. The primary concern of the FDA is to insure the safety of the subjects on the clinical trial. Although they need a sound argument that the proposed therapeutic could be efficacious, as long as the toxicology studies indicate that the proposed human clinical exposures have a sufficient safety margin, the FDA will typically allow the study to be conducted. Clearly, the choice of the preclinical efficacy model is critical. As mentioned previously, the costs associated with conducting the studies required to file an IND for a new chemical entity are minimally 1.5 million dollars. For a company to choose to proceed with these studies, they must believe that they have sufficient evidence from the preclinical efficacy studies to warrant such spending. In the absence of having a validated model, companies often will evaluate the efficacy of the new drug product in multiple *in vitro* and animal models. As an example, for acute focal stroke, studies are typically done in both permanent and transient ischemia models in multiple species. Investigators are trying to get a sense of the breadth of activity of the new drug and to determine if the exposures that were efficacious in one species also provided efficacy in another. Studies will look at the dose responsive nature of the efficacy, as well as the time dependence between when the drug is given versus the induction of the model. For example, if the drug is given before or at various times post stroke.

The choice of the preclinical efficacy endpoint is typically matched to what the FDA has required of other drugs under clinical development. There are normally accepted clinical endpoints for each disease state, whether they be survival or scoring based on a standardized scoring scale or system. Quite often the measures that can be obtained from the preclinical studies cannot fully match those for the clinical studies, and surrogate endpoints are used. The evaluation of how the subjects perceive their condition cannot be evaluated in animal models, but this typically is a critical element for evaluating the recovery of human subjects. Using acute focal stroke as an example again, researchers will evaluate the function of rats post stroke by watching their movement and ranking their performance using a pre-defined scale.

In the case of secondary pharmacology, toxicology, pharmacokinetics and drug metabolism and chemical manufacturing, the preclinical endpoints needed are well defined by the FDA. There are numerous guidance documents published by the FDA that explain what studies need to be done and what endpoints need to be measured.

Defining Clinical Endpoints

The clinical endpoints are chosen based on the need to obtain regulatory approval from the FDA. Because of this, when choosing a clinical endpoint, most investigators first evaluate what endpoints were allowed by the FDA for other drugs in development for that specific disease. Whenever possible, clinical endpoints will be chosen that companies are reasonable sure will be accepted by the FDA. It is possible to reach agreement with the FDA on novel endpoints, but this is a much bigger regulatory challenge. Once again, an effect will be sought at a given exposure based on the preclinical study results. As detailed in Table 2, for most drugs the clinical trial work begins in Phase 0 or 1 with dosing in normal subjects. These studies are designed to evaluate the pharmacokinetics and tolerability of the new drug

product. The results of these studies will be compared with the preclinical data to see if the intraspecies scaling accurately predicted the results in people. The next phase of clinical testing is Phase 2, which involves the first evaluation of trends for efficacy in people with the disease. These studies are intended to allow an assessment of both tolerability and exposure in the target disease population while simultaneously conducting initial evaluation of the potential efficacy endpoints to be used in Phase 3. These Phase 2 trials will provide the basis for conducting statistical analyses intended to determine the size of Phase 3 trials that will be needed to demonstrate statistically significant efficacy based on the clinical endpoints chosen. Phase 2 is also the time to evaluate multiple potential clinical endpoints and decide which are optimal for use in Phase 3. Phase 3 clinical studies are the pivotal efficacy studies required by the FDA for approval when companies file a New Drug Application (NDA), seeking permission to sell their product. Companies have the option of requesting a special protocol assessment (SPA) from the FDA before the start of the Phase 3 trials in order to reach agreement beforehand on how the trials will be conducted, what the clinical endpoints will be, and how the statistical analysis will be handled. If agreement is reached on the SPA review, then if the data meets expectation the FDA is obliged to accept the results as definitive. If a company does not elect to submit an SPA, then the adequacy of the chosen clinical endpoints can be a matter for discussion at the time of the NDA submission, and may preclude the FDA giving approval.

Examples of Translational Research Effort

Nitrones: Their properties and early studies indicating their therapeutic potential

We have thoroughly reviewed the subject of nitrones as therapeutics earlier [26] and therefore this section will be a summary of the most important aspects of this class of synthetic chemicals and the early studies indicating their potential as novel therapeutics. Figure 1 shows the basic nitron structure and the free radical “spin-trapping” reaction which brought attention to this class of compounds that have been used extensively in the last several years. Figure 1 also shows the structure of PBN (α -phenyl-*tert*-butylnitron), which has been used widely as a spin-trapping compound, and the structure of the PBN derivative 2,4-disulfonylphenyl-*tert*-butylnitron which was called NXY-059 as an experimental drug used in preclinical and clinical trials for acute ischemic stroke. This will be discussed later.

Nitrones; Their use in Chemical Reactions to use in Experimental Animals. It is instructive to summarize the experimental research history that led to the realization that PBN-nitrones began to be considered as novel therapeutics. As we have reviewed in detail previously [26], nitrones began to be used in chemical reaction systems to trap free radicals thereby stabilizing the radical to where it could be characterized by various methods including Electron Paramagnetic Resonance. Thus taking advantage of the reaction shown in Figure 1 free radicals with extremely short life times that occurs in chemical reactions could in principle be captured thus extending their lifetime such that they could be identified and characterized. Within a few years after the strictly chemical applications of spin-trapping had begun in the late 1960s [66, 67] there began in the mid 1970s to be a few laboratories, ours included, where the spin-trapping technique began to be applied with success in biochemical systems such as in the trapping of radicals in rat liver microsomes metabolizing the toxin CCl_4 [68]. Their use in biochemical studies then began to be widely used in many laboratories. Very soon thereafter the use of spin traps to study free radicals *in vivo* in some biological systems began in the mid 1980s as for example their use in the detection of free radicals in γ -irradiated mice by McCay's group [69]. At about the same time the first reported use of PBN as a pharmacological protective agent in experimental animals appeared. Novelli showed that PBN was active in protecting rats from traumatic shock caused by confining the animals in a rotating drum or by injecting them with LPS [70–72].

The protective effects of PBN, if administered prior to challenging the rats with LPS, was soon confirmed and extended by 2 other laboratories [73, 74].

Experiments that lead to Nitrones being used in Stroke. The confluence of several fortuitous events and technological advancements led to our laboratories making key discoveries that brought about the extensive commercial development of a PBN derivative for the treatment of Acute Ischemic Stroke. These have been summarized in detail previously [26] therefore only major key events that contributed to making this happen will be emphasized in this report.

The following major factors were very important for us in making the discoveries that eventually lead to the commercialization of a PBN derivative (NXY-059) for the treatment of stroke. First, was our early pioneering focus on and demonstrations that brain is particularly vulnerable to oxidative damage [75–77]. Second, was our development of the salicylate method for an *in vivo* trapping agent of hydroxyl free radicals [78]. Third, was the development of the Mongolian gerbil as an excellent experimental stroke model by Carney and colleagues [79] and their desire to collaborate with us. Finally, this then made it possible to use the salicylate trapping technology to rigorously demonstrate that ROS was formed in the reperfused ischemic regions of the stroked brain [80]. This then confirmed in brain the basic controversial concept, first proposed by McCord and colleagues [81, 82], that reperfused ischemic tissue produces ROS.

Major Discovery from Failed Experiment. With the clear demonstration in hand that reperfused brain produced ROS, specifically hydroxyl free radicals, in the ischemic regions we rationalized that possibly secondary free radicals were formed and that these could be trapped using PBN. Experiments were designed to test this concept but no PBN trapped free radicals were observed using Electron Paramagnetic Resonance (EPR). In order to explain these negative results we rationalized that this was due to the fact that if any PBN-trapped radicals were formed the spin-adduct nitroxyl radical would undergo reduction to its non-paramagnetic state by mitochondrial reductants [83] and therefore rendered EPR-silent. Thus even though this experiment failed, nevertheless in the course of these experiments we made an important discovery, namely that PBN protected the brain from damage caused by stroke even if the PBN was administered after the brain was reperfused [84–86]. This serendipitous novel observation was the key finding that lead to a large commercial development effort to develop a novel nitrone for the treatment of acute ischemic stroke. This effort is briefly summarized in the next section.

Nitron NXY-059 for Treatment of Acute Ischemic Stroke

The discovery that PBN showed protection from experimental stroke even if given up to one hour after the stroke opened up considerable interest in the potential of the PBN-nitrones in neurodegenerative diseases and other age related diseases. And to that end we began to explore licensing the technology to major pharmaceuticals companies but we found that they wanted more basic observations on the PBN-nitrones and more in depth knowledge of the broader implications. In the course of investigating other avenues to further develop our discovery we found an angel investor who recognized the value of the discovery for possible treatment of age related diseases and aging and this made it possible to found Centaur Pharmaceuticals, Inc to help develop the technology. From that time on significant commercial research and development activity was expended with the goal of developing nitrone-based therapeutics to treat age related diseases with a special focus on stroke. Some of the most important milestones in that effort are summarized in Table 3.

We have summarized and described in detail the preclinical [87] and clinical aspects [26] of this effort previously and therefore only major milestones will be discussed here. A major

event occurred when a co-development relationship was made with Astra (later to become AstraZeneca) that made it possible to significantly speed up the research and development effort. From this partnership and through the research that Astra funded, the experimental drug NXY-059 (2,4-disulfonylphenyl-*tert*-butylnitron), which is a PBN derivative, was chosen as a Candidate Drug to develop for the treatment of stroke. From that time forward through the clinical development phases the major thrust was funded and spearheaded by Astra and then AstraZeneca. NXY-059 was found to have no safety issues at all in the phase 1 and phase 2 trials where it was administered IV at very high levels. In the SAINT-I trial which involved 1700 patients, NXY-059 showed significance in the Modified Rankin behavioral test but in the SAINT-II trial which involved 3200 stroke subjects there was no significance. This was a very disappointing result. The time window for the trial was set at 6 hours post stroke.

Many critiques have been published regarding these widely followed clinical trials and various reasons as to why the drug failed [26]. However one that has not been discussed, and in the authors opinion is a major reason, is that MRI screening of patients as an inclusion criteria for the efficacy trials as well as subsequent MRI follow-ups post treatment would have led to finding a select group of subjects that may have shown significant benefit from NXY-059. Unfortunately in 2003 when these trials began MRI technology was not widely available.

Nitrones as Anti-Cancer Agents

Studies in our laboratories have shown that PBN and related nitrones have anti-cancer activity. In the past two decades there have been numerous studies that indicated nitrones may have anti-cancer effects [26]. For example it was established that PBN and related nitrones diminished the effect on CCl₄-induced hepatotoxicity, when administered before exposure to this hepatotoxin [88–91]. CCl₄ is a known carcinogen and thought to initiate carcinogenesis by the formation of free radical metabolites. Definitive proof that PBN and related nitrones have anti-cancer activity was demonstrated in a study which showed that PBN administration in the drinking water significantly decreased the size of pre-neoplastic nodules [92] and decreased the number of adenomas and essentially prevented carcinoma formation in a choline-deficient rat hepato-carcinogenesis model [93]. We also found that PBN significantly inhibited the formation of reactive oxygen species resulting from choline deficiency induced carcinogenesis [94], and provided evidence that PBN acts as an anti-cancer agent by enhancing apoptosis of pre-neoplastic cells [92, 93, 95]. Importantly these studies also showed that long-term administration of PBN itself had no carcinogenic activity. That is PBN itself given over a long time period is not a carcinogen. It is important to note that PBN originally was chosen to test as an anti-cancer agent in the choline-deficient hepatocarcinoma model in part because of the overwhelming evidence that ROxS was involved in cancer development in this model [94] and that PBN had potent antioxidant properties in several biological systems. Since we did find that PBN had anti-cancer activity in this model does not necessarily mean that it is due to its antioxidant properties though. It should be noted that α -lipoic acid is an antioxidant and it actually promotes the growth of pre-neoplastic lesions in the choline-deficient model [96] whereas in contrast PBN decreased the number of these lesions and markedly reduces the size of these lesions due to the selective enhanced killing of the cells within these lesions [97]. We showed that the anti-cancer activity of PBN is positively correlated with its ability to suppress iNOS induction and nitric oxide production [97]. It should be noted however that the mechanistic basis of the anti-cancer action of PBN is yet to be completely understood.

We have shown that PBN has anti-cancer activity in other tumor models, including gliomas [98–100] and colorectal polyps [100], particularly if administered prior to and/or continuously during tumor formation. In the glioma study we showed that PBN increased

the survival rate of glioma-bearing rats, reduced tumor growth (as measured by anatomical/morphological magnetic resonance imaging (MRI)), as well as inhibited angiogenesis (as detected and assessed by MR angiography (MRA) [98]. Recently we demonstrated that the 2,4-disulfonylphenyl derivative of PBN, which we call OKN-007 (also known as NXY-059), has anti-glioma activity and increases survival rates (see Fig. 2, panel 'h'), when administered orally as a post-tumor therapeutic agent [34]. OKN-007 caused a reduction in tumor volumes (see Fig 2) as assessed by MRI [34]. OKN-007 was also found to alter characteristic molecular events associated with tumor development, such as decreasing the levels of (1) c-Met, a marker for tumor cell invasion, (2) iNOS (inducible nitric oxide synthase), a marker for tumor-induced inflammation, (3) and VEGFR2 (vascular endothelial growth factor receptor 2), a marker for tumor-induced angiogenesis [34], which are all over-expressed in gliomas.

It has been shown that in addition to initiating inflammation [101], over-expression of iNOS can also up-regulate tumor angiogenesis [102] and induce mutations and stimulate tumor growth and/or metastasis [103, 104]. It is well known that NO is involved in inflammation and carcinogenesis [97, 105], including colon carcinoma growth and metastasis [106] and pancreatic cancer pathogenesis [107]. The inhibitory effect of nitrones on iNOS may result in decreased NO levels which may affect tumor growth. It has also been found that OKN-007 (NXY-059) was capable of trapping hydroxyl radicals more efficiently than PBN or 2-sulphophenyl-N-tert-butyl nitron (S-PBN) [108] which may contribute to the free radical scavenging capability of OKN-007 as an additional mechanism. Other exogenous antioxidants have also been found to have inhibitory effects on angiogenesis or induce apoptosis, which would both dramatically alter tumor cell proliferation. The antioxidant compound ursolic acid was found to decrease the angiogenic factor, VEGF, NO, and proinflammatory cytokines in mice implanted with metastatic B16F-10 melanoma cells [109]. Other antioxidants have been able to induce apoptosis, such as saponines (found in a *Gynostemma pentaphyllum* extract) in C6 rat glioma cells [110], *Origanum Majorana* in human lymphoblastic leukemia Jurkat cells [111], and Marchantin A (a cyclic bis (bibenzyl ether) isolated from the liverwort *Marchantia emarginata* subsp. *Tosanna*) in human MCF-7 breast cancer cells [112].

In conclusion based on our preclinical studies and its demonstrated human safety studies [113] we think that OKN-007 has good potential as a therapeutic agent for human gliomas. Active preclinical research is now underway in preparation for eventual clinical trials using OKN-007 for the treatment of glioblastoma.

Nitrones as Therapeutics for Other Indications

Nitrones have been shown to be active in many experimental models where reactive oxidant species are involved as we have summarized earlier [26]. However it is important to note that research on nitrones in combination with NAC (N-Acetyl-cysteine) to prevent noise-induced hearing loss has progressed rapidly in the last few years [26, 114]. In addition increased activity has been ongoing in nitrones in experimental models of light-induced damage to eyes affecting vision [115–117]. Other indications that appear to be promising for using the nitrones as therapeutics based on early preclinical research include traumatic brain injury and inflammatory skin conditions.

Conclusions

There is ample mounting evidence that ROxS are exacerbated in inflammatory processes, many pathological conditions and underlying processes of chronic age-related diseases. Therefore there is increased expectation that therapeutics can be developed which act in some fashion to suppress ROxS and therefore ameliorate the condition. This has turned out

to be more difficult than at first expected. It appears that the difficulties arise for several reasons. One possible reason is attempting to use antioxidants that may not act operationally *in vivo* as antioxidants on the processes in question. This may be due to a number of reasons including bioavailability of the antioxidant into the selected target tissue region. Another possibility is the fact that the selected antioxidant may quell the ROxS in question in the target tissue region but still not be effective. This could be due to the fact that the ROxS may just accompany but not really be causative in the disease process. Viewed strictly from a kinetics perspective the disease process may or may not depend upon one or perhaps several key events involving ROxS. If key events in the disease process does depend on ROxS-mediated events then if the therapeutic quells these ROxS it may be expected to be effective. Therefore viewed from this perspective preclinical models should be focused to test these assumptions and early clinical trials should be designed to evaluate these endpoints if possible also. If the clinical endpoints enhance the validity of the preclinical models this would bring enhanced confidence in the therapeutic in question.

Acknowledgments

R. A. Floyd is Merrick Foundation Chair of Aging Research. We acknowledge the Following research grants; Office of Naval Research grant N00014-08-1-0484, Oklahoma Applied Research Support (OARS) grant AR09.2-010 and Army Breast Cancer Research concept grant W81XWH-09-1-0352 to RAF; Oklahoma Applied Research Support (OARS) grant AR052-04 to RAT; and NIH R01 AG031553 to KH.

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Nitrones and Their Properties

Nitronium functionality

Specific Nitrones

A

PBN
α-phenyl-tert-butylnitronium

B

NXY-059
OKN-007
2,4-disulfonylphenyl-tert-butylnitronium

Figure 1.

Presentation of the nitronium functionality and the “spin-trapping” reaction where an unstable free radical reacts with a nitronium to form a relatively more stable nitronium spin-adduct. Also presented are the chemical structures of PBN and the PBN derivative 2,4-disulfonylphenyl-tert-butylnitronium which was called experimental drug NXY-059 in the stroke research and development effort and is now called OKN-007 as an anti-cancer therapeutic.

Figure 2.

OKN-007 treatment of C6 rat gliomas. (a–c) Representative T₂-weighted MR images of C6 glioma-bearing rat brains at days 7, 16 and 20 following intracerebral implantation of C6 rat glioma cells. Tumors are outlined. (d–f) Representative T₂-weighted MR images of an OKN-007 treated rat with a C6 glioma at days 7, 24 and 36 following cell implantation. (g) Tumor volumes at last time point (days 17–21 for non-treated rats (n=5), and days 36–40 for OKN-007 treated rats (n=5)). There is a statistical difference when comparing the 2 groups (p<0.05). (h) Percent survival for non-treated (n=5) and OKN-007 treated rats (n=5) with C6 gliomas, with a statistical significance when comparing the 2 groups (p<0.05).

Table 1

Typical IND Studies for an oral therapeutic required for IND submission intended for clinical use for up to 14 days

Studies	Approximate Duration (months)	Approximate Cost (USD)
Primary Pharmacology		
<i>In vitro</i> efficacy	1	5,000
Animal model: Dose response	2	15,000
Animal model: Time dependence	2	15,000
Secondary Pharmacology		
Irwin neurological assay	1	18,000
GI transit assay	1	10,000
Receptor binding with HERG	1	7,000
Conscious dog cardiovascular	2	120,000
Dog respiratory function	2	120,000
Pharmacokinetics and Drug Metabolism		
Rat pharmacokinetics	1	26,000
Dog pharmacokinetics	1	23,000
Rat 14-Day toxicokinetics	1	20,000
Dog 14-Day toxicokinetics	1	20,000
Protein binding	1	15,000
Dosing solution analyses for GLP toxicity studies	1	20,000
Bioanalytical methods validation	2	40,000
Mouse, rat, dog and human <i>in vitro</i> metabolism	2	20,000
Toxicology		
Acute Rat Toxicity	2	15,000
GLP 14-Day Rat Toxicity	3	145,000
GLP 14-day dog toxicity	3	210,000
Ames assay	1	6,500
CHO chromosome aberration	1	31,000
In vivo rat micronucleus	2	28,000
Chemical Manufacturing and Control		
API manufacture for GLP studies	2	67,000
GMP API manufacture for clinical studies	3	160,000
API Stability studies	Ongoing	30,000
Reference standard certification	1	24,000
Active agent forced degradation and method validation	2	30,000
Polymorph study	1	100,000
Drug product manufacturing	3	55,000
Drug Product stability	Ongoing	55,000
Drug Product analytical method development and validation	2	30,000

Studies	Approximate Duration (months)	Approximate Cost (USD)
Total Costs:		1,480,500

Table 2

Endpoints for Phases of Clinical Development

Phase of Development	Typical Endpoints
Phase 0	Pharmacokinetics and tolerability of very low doses in normal subjects
Phase 1	Pharmacokinetics and tolerability of doses in range of predicted exposures needed for efficacy in normal subjects
Phase 2	Preliminary measures of efficacy and tolerability in patients
Phase 3	Pivotal efficacy measures and tolerability in patients

Table 3**Major Stages in Commercial Development of NXY-059 for Treating Stroke**

1.	Basic Discovery that PBN protected in Experimental stroke – December 1988
	Discovery patented, basic studies continued, companies contacted to license technology
2.	Centaur Pharmaceuticals, Inc. founded – March 1992
	Angel investments made possible, CEO choice made
	Company hires employees, began experiments to add value, explore extent of IP potential
3.	Centaur and Astra made co-development relationship – June 1995
	Ramped up studies in stroke, ramped up medicinal chemistry and for other indications
4.	NXY-059 chosen as candidate drug for stroke-1996
5.	NXY-059 undergoes extensive toxicology and Phase I and II clinical trials-1996–2003
	Tested safety in normal patients and stroke patients and dose-ranging studies
6.	Renovis buys Centaur Assets - 2002
	Renovis and AstraZeneca continue NXY-059 development
7.	NXY-059 phase III (SAINT-I) efficacy trial begins-May 2003
8.	NXY-059 SAINT-I trial shows efficacy in 1700 patients
	Significance in Modified Rankin Test – May 2005
9.	NXY-059 SAINT-II trials begins for 3200 patients-July 2005
10.	Combined SAINT -I and SAINT -II trial showed NXY-059 not effective-October 2006