Chemical Research in Toxicology

Review

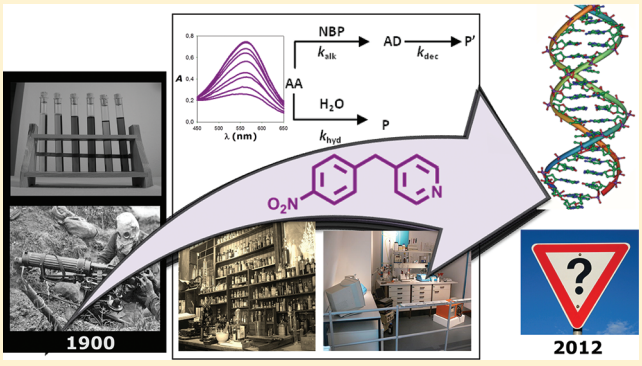
pubs.acs.org/crt

Potential of the NBP Method for the Study of Alkylation Mechanisms: NBP as a DNA-Model

Rafael Gómez-Bombarelli, Marina González-Pérez, Emilio Calle, and Julio Casado*

Departamento de Química Física, Facultad de Ciencias Químicas, Universidad de Salamanca, Plaza de los Caídos, 1-5, E-37008 Salamanca, Spain

ABSTRACT: Alkylating agents are considered to be archetypal carcinogens. One suitable technique to evaluate the activity of alkylating compounds is the NBP assay. This method is based on the formation of a chromophore in the reaction between the alkylating agent and the nucleophile 4-(*p*-nitrobenzyl)pyridine (NBP), a trap for alkylating agents with nucleophilic characteristics similar to those of DNA bases. NBP is known to react with strong and weak alkylating agents, and much insight into such alkylation mechanisms *in vivo* can be gained from kinetic study of some alkylation reactions *in vitro*. Since 1925, the NBP assay has evolved from being a qualitative, analytical tool to becoming a useful physicochemical method that not only allows the rules of chemical reactivity that govern electrophilicity and nucleophilicity to be applied to the reaction of DNA with alkylating agents but also helps to understand some significant relationships between the structure of many alkylation substrates (including DNA) and their chemical and biological responses. Given that advances in this area have the potential to yield both fundamental and practical advances in chemistry, biology, predictive toxicology, and anticancer drug development, this review is designed to provide an overview of the evolution of the NBP method from its early inception until its recent kinetic−mechanistic approach, which allows the pros and cons of NBP as a DNA-model to be analyzed. The validity of NBP as a nucleophilicity model for DNA in general and the position of guanosine at N7 in particular are discussed.



CONTENTS

1. INTRODUCTION

Alkylating agents are considered to be archetypal carcinogens. Certain alkylating agents are primary carcinogens, which do not require metabolic activation to exert their biological effects, whereas most frequently a previous bioactivation step is required.[1] Almost all the heteroatoms in the double helix have the potential to become alkylated. The preferred sites of alkylation in duplex DNA depend strongly on the nature of the alkylating agent. For example, the *N*7 position of the guanine residue is the most nucleophilic site on DNA bases and is a favored site of reaction for almost all small, freely diffusible alkylating agents.[2] The electrophilic groups in many alkylating agents are inherently labile and undergo relatively rapid hydrolysis, which renders them inactive.

Because of their DNA-damaging ability, alkylating agents were the first compounds identified to be useful in cancer chemotherapy. Many antineoplastic drugs routinely used today in cancer therapy are alkylating agents, including nitrogen mustards such as melphalan, chlorambucil, cyclophosphamide, and ifosfamide; nitrosoureas such as carmustine, lomustine, and streptozotocin; alkyl sulfonates; thioTEPA; and, in a broader

Received: February 16, 2012
Published: April 5, 2012

   dx.doi.org/10.1021/tx300065v | *Chem. Res. Toxicol.* 2012, 25, 1176−1191

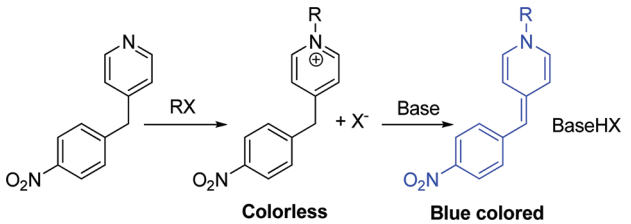
sense, even cisplatin and other platinum complexes. Several of these drugs have at least two reactive sites and are able to form cross-links between DNA strands, preventing their separation for processes such as replication and RNA transcription.

One suitable simple and highly selective technique to determine the activity of alkylating compounds is the colorimetric NBP method. The nucleophile 4-(*p*-nitrobenzyl)pyridine (NBP), a trap for alkylating agents with nucleophilic characteristics similar to those of DNA bases,[3,4] is known to react with strong[5−7] and weak[8−12] alkylating agents, and much insight into such alkylation mechanisms *in vivo* can be gained from the kinetic study of this reaction *in vitro*.[13−15] Accordingly, here we provide a brief overview of the main aspects related to the NBP chemistry because (i) almost all of the heteroatoms in the double helix have the potential to be alkylated, such that the physical chemistry of DNA damage is of crucial importance in diverse fields such as medicinal chemistry, toxicology, and biotechnology;[16−18] (ii) NBP is often quoted as a model for DNA. This assumed relationship is usually taken as a premise and applied with the aim to establish correlations of the response in the NBP assay with the results from other assays or the effects *in vivo*;[7,19] (iii) to our knowledge, apart from some analytical aspects of the NBP method, there is a lack of a general review on aspects as relevant as the kinetics and mechanisms of alkylation reactions, or correlations between the chemical reactivity of alkylating agents (NBP method) and their carcinogenicity.

2. DEVELOPMENT OF THE NBP ASSAY

2.1. Early Work. Early investigations addressing the detection of alkylating agents were performed in the beginning of the 20th century. A precursor of the NBP test was developed by Decker, using α and γ-benzylpyridine,[20] and the first application of 4-(*p*-nitrobenzyl)pyridine to detect alkylating agents was implemented in 1925 by Köenigs et al.[21] This initial attention paid to the NBP assay was a consequence of the interest in the reactivity and detection of the vesicant, mutagenic, and carcinogenic compounds used in chemical warfare, and indeed, the US military was the first to try to correlate color intensity with the reactivity of the alkylating agent and also to propose a mechanism for the reaction (Scheme 1). Epstein et al. improved the accuracy of the method

Scheme 1. Alkylation Reaction of NBP and Development of Color



and applied it for quantitative determinations.[3] This seminal work has been cited profusely, and some authors refer to the NBP assay as the Epstein test. In general, the experimental procedure is based on spectrophotometric measurement of the color intensity of alkylated NBP after basification (Scheme 1). The colored adduct obeys the Lambert−Beer law, and the absorbances measured can therefore be correlated with the concentration of adduct. Since the chromophore is unstable in alkaline medium, the measurements must be taken rapidly.

Depending on the solubility of the alkylating agent in water, two general modifications of the test have been used: (i) In aqueous media, solutions of the alkylating agent containing phthalate as a buffer are mixed with NBP in acetone, heated at 100 °C for 20 min, and then cooled in an ice−water bath. Potassium carbonate in water/acetone (1:4) is used as the base. Many investigators have used this form of the assay with minor modifications.[19,22−48] (ii) For water-insoluble compounds, methyl ethyl ketone is used as a reaction medium instead of water. Since the alkylation reaction proceeds slowly in organic media, it is allowed to run for 45 min. Acetone is added, and after cooling, the base triethylamine is added. Higher temperatures are used to dissolve the reagents in less volatile solvents such as acetophenone[49] and ethylene glycol.[50] Ethylene glycol has the additional advantage of preventing the formation of separate organic and aqueous phases, as often occurs.[22,51−62] The method introduced by Preussman[63] uses glycol monomethyl ether as solvent, and piperidine has been used extensively as a base.[64−70]

2.2. Biological Samples. With the advent of alkylating antineoplastic agents, interest in the NBP assay as an analytical tool for biological samples increased. The ability to detect these drugs in biological fluids, however, required some modification to the method: centrifugation, precipitation, and chemical[23,71,72] or thermal denaturation[73] to separate possible interfering constituents of biological fluids and changes in the solvents and buffers with a view to improving sensitivity[74] and reproducibility.[73,75]

Friedman and Boger added further modifications to the original method, by extracting the colored product with ethyl acetate, using NaOH as a base.[73] This approach became the most commonly used method for the estimation of alkylating agents in biological samples.[4,76−87] Other variations include gassing the sample[88−91] or changes in the solvents,[60,92−95] often aimed at avoiding the extraction step.[96] Skibba and Collins have also proposed some modifications for the detection of antineoplastic nitrogen mustards.[97] Additionally, after following the kinetics of the NBP alkylation reaction by a variety of alkylating agents,[98−100] Kawazoe concluded that temperature and pH must be carefully monitored[101] because of the lability of the reactants, and a pH above 12.5 is needed for quantitative dissociation of the adduct. After these works, small modifications have been applied and then only on specific occasions. An additional set of variations exists with regard to the use NBP as a developing reagent in thin-layer chromatography, where NBP is sprayed onto the plate, after which a base added.[102−113]

2.3. Alternative Assays. Several, less successful, at least in terms of widespread use, alternatives to NBP have been proposed, e.g., 8-quinolinol for the detection of bifunctional nitrogen mustards.[114] 2,6-Dibromo-*N*-chloro-*p*-quinone imine and trichloro-*p*-quinone imine show selectivity for the detection of compounds containing P═O and P═S bonds.[115] Substitute chromogens such as that of *p*-nitrothiophenolate have also been used,[116] with lower detection limits and better color stability.[49] Fuchsin has also been proposed as an alternative for the study of epoxide hydrolases since it gives better results for the less reactive oxiranes.[117,118] On studying the stability of alkylating agents, NBP is appropriate only for those with intermediate reactivity.[29] A colorimetric assay based on nicotinamide has been proposed, which allows the use of more biomimetic conditions and better color stability.[40] The rate constants are also comparable to those for NBP.[37]

N-Mercaptoimidazole (MMI) has also been used to test S_N2 reactivity. However, the reaction must be monitored by

dx.doi.org/10.1021/tx300065v | *Chem. Res. Toxicol.* 2012, 25, 1176−1191

^{1}H NMR spectroscopy since the assay is not colorimetric. In addition, MMI has two reactive sites: *N*- and *S*-atoms. Reactions with H_2O or trifluoroacetate are often used as a measure of S_N1 alkylating activity and thus as probes for O-alkylation.[119−121]

3. NBP AS A DNA-MODEL

NBP has often been cited as a model for DNA in general and for the *N*7 position of guanine in particular.[4,72,122] This assumed relationship is usually taken as a premise, and the technique is applied with the aim of establishing correlations of the response in the NBP method with the results from other assays or the effects *in vivo*.[19] Below, we explore whether NBP might be a valid model for DNA and thus the validity of the above premise.

DNA is a very complex polymer, with a variety of *N* (endo- and exocyclic) and *O* (carbonyls and phosphates) nucleophilic positions that may react with alkylating agents simultaneously, affording different reaction products. Even nucleotides have several reactive sites. This, together with other factors such as the low solubility of nucleobases or the cost of commercial DNA, and above all the difficulty of monitoring the reagents and products, makes kinetic study of DNA alkylation very difficult and encourages the use of models.

Whereas the multiplicity of reactive sites makes it impossible to focus on a specific reactive site on polymeric DNA, experimental work has suggested that guanosine (i.e., its *N*7 endocyclic nitrogen) is the most nucleophilic position in DNA.[123,124] As a consequence, the majority of adducts formed between alkylating agents and DNA are *N*7-guanine adducts,[2] and the ability of alkylating agents to modify this DNA site is considered evidence of genotoxicity.

The alkylation of *N*7 guanine has several possible consequences: (i) cross-linkage of two adjacent guanine residues in the case of bifunctional agents; (ii) base mispairing, which produces base-pair substitution (usually GC→AT transitions) or strand breaks; and (iii) depurination; *N*7 alkylation causes cleavage of the imidazole ring and guanine residue excision. Mutagenesis by depurination may occur by insertion or deletion.[125] In addition, the apurinic site formed may result in a highly genotoxic strand break. Thus, the reaction of *N*7 position of guanine and of the reactive site in NBP is in fact a valid model for the most reactive sites in DNA.

3.1. Nucleophilicity of DNA. Despite the complexity of DNA, many efforts have focused on applying the basic rules of chemical reactivity that govern electrophilicity and nucleophilicity for the alkylation reaction of DNA by alkylating agents (AA). One of the most simple scales of nucleophilicity/electrophilicity and by far the one most frequently applied to the reaction of DNA with alkylating agents is that developed by Swain and Scott (eq 1).[126]

$$\log(k_N/k_o) = (n_N - n_o)\cdot s \quad (1)$$

This free-energy relationship[127] relates the pseudo first-order reaction rate constant (k_N; in water at 25 °C) of a reaction, normalized to the reaction rate of a standard reaction with water as the nucleophile (k_o), to a nucleophilic constant, (n_N and n_o), and a substrate constant, *s*. These depend, respectively, on the nature of the nucleophile and the sensitivity of the substrate to nucleophilic attack.[128] Since the reaction is assumed to take place in aqueous solution, no conclusions can be drawn regarding the role of the solvent. More powerful contemporary approaches, such as those of Ritchie[129,130] or Mayr-Patz,[131] have been applied less frequently to the reaction of DNA with electrophiles.

Table 1. Swain−Scott Nucleophilicity Constants for Some Nucleophiles of Biological Interest

nucleophile	formula	*n*
DNA[211]		2.5−2.6
monomeric DNA[212]		5.4
*N*7-guanine[211]		3.5−3.6
*O*6-guanine[211]		0.5−1.5
DNA phosphate[133]		1
-RS- and-RSH in cysteine (avg)[211]		∼5
inorganic phosphate	$H_2PO_4^-$	2.5
acetate[128]	CH_3COO^-	2.72
thiosulphate[128]	$S_2O_3^{2-}$	6.36
water[128]	H_2O	0.00
azide[128]	N_3^-	4.00
hydroxide[128]	HO^-	4.20
iodide[128]	I^-	5.04
aniline[128]	$C_6H_5NH_2$	4.46
pyridine[190]	C_6H_5N	4.27
NBP[212]	$C_{12}H_{10}N_2O_2$	3.5

Table 1 shows the Swain−Scott nucleophilicity constants for some common nucleophiles. The values reported show that NBP and *N*7-guanine have almost identical *n* values and therefore similar nucleophilicity. In contrast, pyridine and aniline have somewhat higher values. More importantly, these values are very different from those for DNA: double-stranded DNA has much lower nucleophilicity, whereas its monomeric form has much higher values. However, the values for either single-stranded or duplex DNA are difficult to compare with those of monomers, and such conclusions must be taken carefully since (i) steric hindrance is not explicitly taken into account in the Swain−Scott equation, and hence, steric effects are seen as deviations from the ideal behavior rather than genuine effects; (ii) DNA has a variety of different nucleophilic sites, with very different reactivities; and (iii) electrophiles such as carbocations, which include most S_N1-acting alkylating agents, deviate significantly from ideal Swain−Scott behavior.[128]

3.2. Site Selectivity. An interesting consequence of the multiplicity of reactive alkylation sites is that certain alkylating agents show particular preference for some nucleophilic positions: for instance, carbocations react with oxygen atoms, whereas other electrophiles, such as oxiranes, mainly react with nitrogen nucleophiles. The factors governing this reactivity are a combination of steric and electronic effects.[132,133]

Several approaches have been proposed to explain this complex behavior (Table 2):

- In terms of the Swain−Scott equation, electrophiles with low *s* values are less sensitive to the greater *n* value of nitrogen nucleophiles and hence preferentially react at *O*-sites. Alkylating agents with high *s* values are sensitive to the increased nucleophilicity and react with *N*-sites.[98,134]
- In terms of molecularity, S_N1-alkylating agents show a greater preference for *O* nucleophilic positions, whereas *N*7-guanine adducts are formed via S_N2 reactions. This view is controversial, and the particular behavior of certain alkylating agents has been putatively interpreted as an unexplained deviation from ideal Swain−Scott behavior rather than as a fundamental difference in the alkylation mechanism.[135]

dx.doi.org/10.1021/tx300065v | *Chem. Res. Toxicol.* 2012, 25, 1176−1191

Table 2. Selectivity in the Formation of DNA-Adducts via Several Approaches

Swain–Scott		molecularity		hard–soft	
AA	site	AA	site	AA	site
low *s*	*O*	S_N1	*O*	hard	*O*
high *s*	*N*	S_N2	*N*	soft	*N*
				small and diffusible	*N*7-Guo

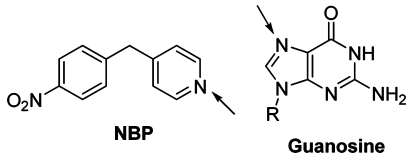
- In terms of the hard–soft reactivity theory,[124] the selectivity observed has been explained as follows. Whereas small, diffusible alkylating agents react with the *N*7 guanosine position, the reactivities at other nucleophilic sites in DNA can be explained in terms of soft–soft and hard–hard selectivity.[2,135,136]

None of these approaches wholly explains the fact that very different adducts are formed in the reaction of DNA with certain kinds of alkylating agents. However, as regards this overview, it perhaps suffices to address the following issue.

3.3. To What Extent Is DNA Modeled by NBP? The first and most important difference between DNA and NBP is their polymeric and monomeric nature, respectively. Large differences in terms of steric hindrance, accessibility, and nucleophilicity also exist. Thus, NBP can hardly be an accurate model for DNA as a whole. It is, however, a valuable model for one particular position.

The reactive site in NBP is an aromatic amine group in the pyridine ring, whose electronic configuration is quite similar to that of *N*7-guanine (Scheme 2), as demonstrated by their

Scheme 2. Guanosine and NBP



almost equal Swain–Scott *n* factors.[137] Steric hindrance is also quite similar in NBP and monomeric guanosine, both sites being fairly unimpeded. However, certain bulky alkylating agents may react with a small molecule, such as NBP, but may be unable to reach the nucleophilic nitrogen positions in DNA due to the much more cramped nature of the macromolecule, which would lead to false positives.

Thus, NBP is a suitable DNA-model insofar as the electrophiles under study show high affinity for the *N*7 position of guanine. That is, for small, diffusible S_N2-reacting alkylating agents the reaction with NBP is very similar to that of DNA, and much insight into the *in vivo* alkylation mechanism can be obtained.[138] In this case, the observed reaction rate is that of the bimolecular alkylation reaction.

In contrast, NBP is a poor model when hard S_N1-reacting electrophiles are studied because these mainly react at oxygen sites *in vivo*. Also, because the NBP test is nonspecific[138] when the reaction kinetics is monitored, in fact it is the formation of the unimolecular carbocation that is followed.

In conclusion, not much insight can be gained from the use of NBP as an alkylation substrate to model the reaction of DNA with alkylating agents that react via unimolecular cleavage or have great affinity for centers other than *N*7 DNA. In these cases, a probe with an *O* alkylation site could offer a better model for *O* alkylation. For the same reasons, aniline is expected to be a better model compound for the *N*2 guanine or *N*6 adenine positions.

3.4. Pros and Cons of NBP as a DNA-Model. Beyond discussion of its reactivity as a DNA-model, other considerations about the (dis)advantages of the NBP test must be taken into account. Apart from the ability to model the nucleophilicity of *N*7 guanosine, the advantages that have encouraged the use of the NBP assay range from its simplicity (UV–vis detection), rapidness, and low costs[77,139] to its sensitivity, reproducibility,[49,97] and versatility.[49] It has also been successfully coupled to *in vitro* metabolic activation systems.

The fact that alkylation products can be monitored selectively by UV–vis spectroscopy has also encouraged the use of NBP in kinetic and mechanistic studies since the use of DNA is much more expensive and involves considerable experimental difficulties.

The NBP assay has several drawbacks that should be kept in mind: (i) NBP is insoluble in water, and aquo-organic mixtures are required, which are very different from *in vivo* conditions (although the dielectric constant of these water/organic mixtures may be similar to that of the major groove of DNA);[140] (ii) occasionally, salt concentrations may be very different from physiological values; (iii) NBP-AA adducts hydrolyze in basic media, which requires fast measurements, or at least for these to be made at equal time intervals;[40,141] (iv) the nonspecificity of the method hinders the use of NBP in the case of mixtures of alkylating agents;[40,78,92–94] (v) its use in acidic media is complicated by the protonation of the nucleophilic nitrogen, whose pK_a must be taken into account; and (vi) the most usual variations of the NBP assay do not take into account bioactivation processes (e.g., oxidation by P450 cytochromes), and accordingly, indirect alkylating agents afford false negatives. The degree of hindrance or benefit introduced by these features depends on the application sought. For instance, in order to correlate kinetic results with *in vivo* activity, the electrophile under study must fulfill certain requirements (*vide supra*), which are not needed for purely analytical applications.

4. USES OF THE NBP METHOD AND ALKYLATION MECHANISMS

4.1. Detection and Quantification of Alkylating Agents. *4.1.1 Detection of Toxic Agents.* As stated above, since its inception the NBP test has been applied for the detection of alkylating agents from a toxicological point of view, that is, to determine whether air or water samples contain alkylating agents, regardless of their nature. Some of these uses have been proposed for applications in warfare. In such work, the NBP test has been used as a qualitative method for the detection of generic alkylating agents: first mustard gas and later other alkylating agents (diethyl sulfate, butyl thiocyanate, benzene sulfonyl chloride, diphenylchloroarsine, and diethyl phosphorofluoridate).

As regards the formation of alkylating agents in foods, Archer and Eng observed that nitrosodiethylamine reacted with NBP to yield an alkylating agent in a nonenzymatic chemical activation system. This approach was also applied to other cyclic and acyclic nitrosamines.[77] Unidentified alkylating nitroso compounds were detected in the reaction of common foods after incubation with nitrite under biomimetic gastric conditions.[142]

dx.doi.org/10.1021/tx300065v | *Chem. Res. Toxicol.* 2012, 25, 1176–1191

The formation of mutagenic chlorination byproducts was addressed in early studies,[28,143] before their identification as halogenated hydroxyfuranones.

Several applications have also involved the detection of organophosphorous pesticides,[144] estimation of insecticide levels on agricultural crop foliage,[145] and the validation of an oxidative treatment for alkylating agents present in residual waters.[146]

NBP has very often been used as a TLC-developing agent, usually to detect organophosphorous pesticides[109,113,115] but also aza-heterocycles, such as diazinon,[147] trichothecene mycotoxins,[112] nitrosoureas,[148] or drugs such as thioTEPA.[104,111]

Addressing the detection of phosgene, a toxic atmospheric pollutant and an industrial chemical, Nakano et al. developed a porous tape impregnated with a NBP solution able to detect 6 ppb of phosgene in air.[139]

The formation of alkylating metabolites of chemicals and drugs has also been addressed: Guengerich et al. used NBP to detect 2-cyanoethylene oxide, an alkylating metabolite of acrylonitrile;[149] Cioli et al. tested the alkylating capacity of a putative metabolite of hycanthone, an antischistosomal drug;[102] and Padgette et al. detected the formation of an epoxide metabolite of 1-phenyl-1-(aminomethyl)ethane hydrochloride.[41]

The NBP test was applied by Fox et al. for the characterization of the oxidative activity of methane monooxygenase purified from *Methylosinus trichosporium* OB3b on haloalkenes. The alkylating ability of the reaction products helped to confirm that epoxides were formed.[150] Similarly, Newman et al. used NBP to capture the short-lived epoxide formed in the oxidation of tricholoroethylene by toluene 2-monooxygenase from *Burkholderia cepacia* G4.[151] Forkert et al. followed the formation of an epoxide from diallylsulfone in their study of CYP2E1 inactivation.[152,153]

4.1.2. Detection of Pharmacological Agents. The NBP assay has been proposed for the detection of antineoplastic alkylating agents in biological tissues in order to determine their pharmacokinetics and pharmacodynamics when administered as a treatment.

Many examples exist: phenylalanine mustards in blood; chlorambucil, sarcochlorine, mannomustine, and others in plasma and urine;[74] mannitol myleran in urine and blood;[50] bis(2-chloroethyl)methylamine in plasma, lymph, urine, or tissue homogenates;[154] melphalan and mechlorethamine in plasma;[97] and 3-[bis(2-chlorethyl)-carbohydrazide]-2,2,5,5-tetramethylpyroline-1-oxyl, a spin-labeled nitrogen mustard,[53] and bioreductive diaziquones.[79]

NBP has often been used as the substrate of choice for the study the metabolic fate of cyclophosphamide[58,155] and the activity of its alkylating metabolites.[76,78,80,156] Mitomycin C,[88−91,157] thioTEPA,[74,86,103,104,111,154,158] and ifosfamide[92−95] have also attracted considerable attention.

Work is still being carried out on updating the NBP method; Dierickx et al. revised the method using some alkylating agents with therapeutic applications (using melphalan, *m*-sarcolysin, chlorambucil, cyclophosphamide, and ifosfamide) in human serum and aqueous media.[141]

4.1.3. Indirect Detection. The NBP test has also been used as a purely analytical tool to study the solvolysis reactions of alkylating agents by monitoring the concentration of unreacted electrophiles.

Compounds such as bis(2-chloroethyl)methylamine (HN2) and bis(2-chloroethyl)amine (nor-HN2),[73] 3-methyl-1-phenyltriazene and some cyclic derivatives,[38] chloroethylene,[31] or epoxy-starch derivatives[159] have been investigated. However, the main application in this field has been the study of epoxide hydrolysis and, in particular, the activity of epoxide hydrolases.[160]

One of the first of these NBP-based colorimetric assays was that of Rink et al., who used a microplate variation to assess the concentration of unreacted epoxide and thus evaluate catalytic activity.[161] This method, based on the work of Miller and Guengerich (who themselves cite the study on chloroethylene by Barbin[31]), has been improved several times.[117,118,162−164] These assays have frequently been applied in the study of epoxide hydrolases obtained from a variety of sources, such as *Streptomyces*,[165] *Sphingomonas echinoides*,[166] *Phanerochaete chrysosporium*,[167,168] *Cupriavidus metallidurans*-CH34,[169] and also to study the oxidative activity of cytochromes[170] or styrene monooxygenase.[171]

4.2. Characterization of Alkylating Agents. Many mutagenic agents afford positive results in the NBP assay (epoxides, lactones, *N*-nitroso compounds, ethyleneimines, halogenated compounds, alkyl sulfonic esters...), whereas many nonmutagens or noncarcinogens show no response.[55]

Thus, NBP was proposed as a reagent for the search for quantitative, semiquantitative, or simply qualitative correlations between alkylating activity and biological effects (carcinogenicity, mutagenicity, toxicity...). Kim and Thomas have suggested that "... the test may prove to be a simple, non-biological indicator of carcinogenic risks",[4] and as such, the NBP assay has been applied as an alert system to assay new compounds whose *in vivo* effects are unknown. These applications can be classified in two groups: those that measure absorbance at a single, fixed time (nonkinetic methods) and those that monitor the alkylation reaction to obtain kinetic and mechanistic information.

4.2.1. Nonkinetic Applications. In nonkinetic applications, the absorbance of the NBP + alkylating agent mixture in an appropriate solvent is measured, after basification, when a certain reaction time has elapsed (*vide supra*). The absorbance at the wavelength of measurement is taken as the quantitative response.

These absorbance values are a measurement of the reactivity of an alkylating agent and have been correlated, more or less successfully, with biological properties such as toxicity, mutagenicity, carcinogenicity, and antitumoral activity, as discussed in the following sections.

Table 3. Mutagenic Events and Target Sequences for Some *Salmonella typhimiurium* Strains

Salmonella strain	reversion event
TA 1536	Frameshifts
TA 98	frameshifts
TA 1538	frameshifts
TA 97	frameshifts
TA 1537	frameshifts
TA 100	base pair substitutions
TA1535	base pair substitutions

4.2.1.1. Correlation of Chemical Reactivity with Mutagenicity, Genotoxicity, and Carcinogenicity. The most common mutagenicity assays, bacterial reverse mutation tests (Ames test), use amino acid-requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations

Table 4. Correlation between Nonkinetic NBP and Mutagenicity

group	compd	Ames test		NBP test	
		−CAS[a]	+CAS	−CAS	+CAS
various[213]	bisphenol A	−	−	−	
	nitrosylated bisphenol A			+	
halogenated compounds[213]	ethylene dichloride	+	+	+	+
	trichloroethylene	−	+	−	+
	tetrachloroethylene	−	−	−	−
	1,2-dibromoethylene	NR	NR	+	+
	p,p-1,1-dichloro-2,2-bis (chlorophenyl)ethane(DDT)	−	−	−	−
	p,p-1,1-dichloro-2,2-bis (*p*-chlorophenyl)ethane(DDD)	−	+	−	−
	p,p-1,1-dichloro-2,2-bis (*p*-chlorophenyl)ethylene(DDE)	−	+	−	+
	p,p-1,1-dichloro-2,2-bis (*p*-chlorophenyl)-2-chloroethylene(DDMU)	−	−	−	−
	p,p-1,1-dichloro-2,2-bis (*p*-chlorophenyl)ethylene(DDNU)	−	−	−	−
Michael acceptors[214]	allyl alcohol	−	−	−	+
	acrylamide	NR	NR	+	+
	acrylic acid	NR	NR	+	+
	acrylonitrile	+	+	+	+
various[214]	diethylnitrosamine	+	+	−	+
	fumaric acid	NR	NR	+	+
	2-furoic acid	−	−	−	+
	2,4-hexadienal	NR	NR	+	+
	caffeine	−	−	−	−
	1-methylhydrazine	−	−	−	+
	1,1-dimethylhydrazine	+	+	−	+
	styrene oxide	+		+	
p-substituted α-methylstyrene oxides[44]	H	+		+	
	Br	−		+	
	Cl	+		+	
	CN	+		+	
	NO_2	+		+	
	phenyl	+		+	
halogenated compounds[55]	neopentyl bromide	−		−	
	pentaerythrityl tetrachloride	−		−	

[a]Chemical Activation System.

(Table 3), which involves the substitution (strains TA100 and TA1535), addition, or deletion of one or several DNA base pairs (TA98, TA1538, TA 1536, TA 97, and TA1537). Acceptable qualitative correlations have been found between the results in nonkinetic NBP assays and mutagenicity in several test systems (Table 4).

Other authors have reported semiquantitative correlations: the mutagenic and alkylating potential followed the same order for some epoxycyclohexanes,[47] a series of three 1,2-dibromo alkanes, and the compounds resulting from their conjugation with GSH.[57]

More importantly, quantitative correlations between NBP alkylating activity and mutagenicity have also been found (Figure 1). These are generally consistent within groups of related chemicals, such as haloallyl compounds,[51,54,172] epoxides,[36,37,47,48] nitrosocarbamates,[173] nitrosoureas,[174] and other compounds.[52]

In addition, it must be taken into account that equal absorption coefficients are usually assumed for all alkylating agents since it is absorbance and not the concentration of the adduct that is measured. This assumption may work within classes of compounds, but attention must be paid to substituents that have strong effects on chromophores, such as carbonyl or diazo groups, and conjugated π systems, such as allyl or aryl groups. Intergroup correlation, however, is often poor,[35,36] and discrepancies in response among different classes of compound have been reported.[32,44,45,67,175−177]

Several explanations for this lack of consistency have been advanced: (i) a higher competition by solvolysis in the case of more reactive compounds;[119] (ii) differences in selectivity toward *N* and *O* nucleophiles;[1,142] (iii) differences in the mutagenic events arising from *O*6 and *N*7 lesions;[119] (iv) the mutagenic test system used was not appropriate;[67] (v) interference by other reactions, e.g., in the case of *N*-methyl-*N*′-alkyl-*N*-nitrosoureas, the effective alkylating agent is formed during the decomposition of nitrosoureas;[178] and (vi) mutagenicity is influenced more intensely than alkylating activity by properties such as polarity,[45] molecular volume,[44] or the degree of unsaturation of aryloxides[177] or glycidyl oxides.[176]

Whereas chemical carcinogens exert their effects through many different mechanisms, the NBP test has also been found to be a good predictor of the carcinogenicity and *in vivo* genotoxicity of alkylating agents (Table 5).

Several investigators have searched for a correlation between genotoxicity *in vivo* and chemical reactivity *in vitro*. Giri et al. studied four aliphatic epoxides that afforded positive results in the NBP assay and also produced DNA strand breaks. The lack of correlation between the response in both assays has been attributed to the different half-lives of the alkylating agents under physiological conditions.[179] Good qualitative and

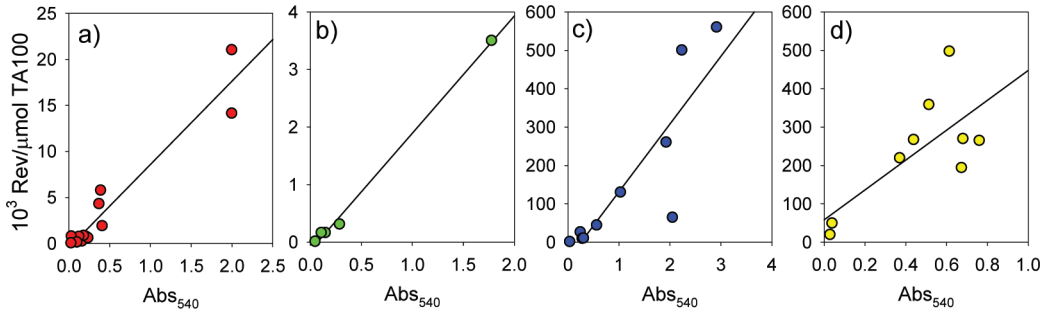


Figure 1. NBP assay. Mutagenicity correlation for some alkylating agents: (a) Propylene oxides;[37] (b) epoxides;[36] (c) haloallyl compounds;[172] and (d) nitrosoureas[174].

Table 5. Correlation between Carcinogenicity, Genotoxicity, *in Vivo* and the Nonkinetic NBP Assay

		genotoxicity	alkylating activity[a]	
	carcinogenicity[a]	*in vivo* Comet assay	+CAS	−CAS
ethylene dichloride	+	+[215]	+	+
trichloroethylene	+	−[216]	+	−
tetrachloroethylene	−	−[215]	−	−
DDT	+		−	−
DDD	+		−	−
DDE	+		+	-
acrylamide	+	+[217]	+	+
acrylonitrile	−	−[218]	+	+
diethylnitrosamine	+	+[219]	+	−
2-furoic acid	−		+	−
caffeine	−		−	−
1-methylhydrazine	+		+	−
1,1-dimethylhydrazine	+	+[220]	+	−

[a]Taken from ref 214.

quantitative correlations between *in vitro* alkylating, mutagenic, and genotoxic properties have been found for a series of allylic compounds as measured by the NBP test, the Ames test, and unscheduled DNA synthesis, respectively.[180]

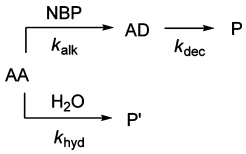
A poor correlation was found between toxicity in mice and leukemic L1210 cells for a series of *N*-nitrosoureas,[181] whereas the agreement between carcinogenicity, mutagenicity, and skin sensitization by very different alkylating agents suggested that the three processes share a common mechanism.[182]

Many alkylating agents have found use as antineoplastic agents since cancer cells have a high division rate, and hence, their DNA is more exposed as compared to healthy tissue. Thus, it would seem that alkylating capacity would be a good predictor of antitumor properties, although this is seldom the case.[65,84,85,183,184]

The nonkinetic NBP assay has also been used for the characterization of the *in vivo* mechanism of the action of chemicals such as alkylhydrazones, which are autooxidized to alkylating agents;[99] atmospheric electrophiles;[185] or [^{18}F]-labeled benzyl fluoride[62] and drugs such as transferrin and albumin conjugates of the clinical alkylating agent chlorambucil[186,187] and bifunctional alkylating agents.[46]

4.2.2. Kinetics of Alkylation: Alkylation Mechanisms. The first efforts to correlate the biological effects of such antitumor activity,[23] toxicity,[23] or tumor incidence[188] with kinetic parameters used common nucleophiles such as water,[23] thiosulfate, or azide,[189] and especially NBP.

Scheme 3. Generic Alkylation Mechanism



A general mechanism for the alkylation of NBP by a generic alkylating agent (AA) can be summarized as shown in Scheme 3: the alkylating agent reacts with NBP, affording a labile adduct (AD), which, in turn, decomposes. Simultaneously, the alkylating agent undergoes solvolysis (in most cases, hydrolysis and also alcoholysis in aquo-alcoholic mixtures[190]) and possibly other deactivation processes such as isomerization. The hydrolysis reaction also refers to rearrangements or any other deactivation process. For simplicity, the catalytic terms in hydrolysis and decomposition have been neglected.

The differential rate equations derived from such a mechanism, when the reaction proceeds through an S_N2 mechanism are

$$\frac{d[AA]}{dt} = -k_{alk}[NBP][AA] - k_{hyd}[H_2O][AA] \quad (2)$$

$$\frac{d[AD]}{dt} = k_{alk}[NBP][AA] - k_{dec}[AD] \quad (3)$$

Their integrated counterparts are

$$[AA] = [AA]_o e^{-(k_{alk}[NBP]+k_{hyd}[H_2O])t} \quad (4)$$

$$[AD] = [AA]_o \frac{k_{alk}[NBP]}{k_{dec} - k_{alk}[NBP] - k_{hyd}[H_2O]} \times (e^{-(k_{alk}[NBP]+k_{hyd}[H_2O])t} - e^{-k_{dec}t}) \quad (5)$$

Depending on the rates of the three processes, three different approaches to the discussion of the kinetic NBP test exist: (i) considering only the alkylation reaction;[8] (ii) also including the parallel hydrolysis;[11] or (iii) taking into account the three processes simultaneously.[10]

Choice of the appropriate approach is of importance, and abundant instances of poor mechanistic perspective in the kinetic application of the NBP test can be found in the literature. For instance, competing hydrolysis and isomerization reactions have often been completely overlooked in the study of epoxides, especially those of polycyclic aromatic hydrocarbons.[26,27,69,191−193] Such assumptions are not unique to oxiranes, and they have also appeared in the study of nitrogen mustards and aziridines,[23] chloroethylene,[31] cyclophosphamide,[194] 3-methyl-1-phenyltriazenes,[38] *S*-(2-haloethyl)-1-cysteine

dx.doi.org/10.1021/tx300065v | *Chem. Res. Toxicol.* 2012, 25, 1176−1191

analogues,[83] antineoplastic agents,[195] haloacetonitriles,[196] *N*-methyl-*N*′-aryl-*N*-nitrosoureas,[175] and phosphoraziridines.[25]

The NBP test has obvious limitations: the lack of selectivity and applicability only to S_N2 alkylating agents, in addition to the fact that correlating the results of the NBP assay with *in vivo* results is sometimes oversimplistic and neglects important processes such as metabolism or DNA-repair systems. Regardless of these flaws, the results obtained in the kinetic NBP test are often the product of an improper kinetic approach rather than anything else. Hence, the presence or lack of *in vivo*−*in vitro*−NBP correlations in these faulty kinetic studies must be taken cautiously.

In the following sections, we discuss the different mechanistic approaches to the kinetic NBP test.

4.2.2.1. Alkylation Alone. If the hydrolysis of the alkylating agent is negligible and the adduct is reasonably stable under the reaction conditions, then only the rate of alkylation is of importance, and the rate equation can be simplified to

$$[\mathrm{AD}]_t = [\mathrm{AA}]_o(1 - e^{-k_{alk}[\mathrm{NBP}]t}) \quad (6)$$

In this case, the alkylating potential of the species can be correlated directly with its alkylation rate constant: the faster the alkylation, the less time the organism has to excrete or deactivate the alkylating agent and the more intense the effects. However, if no alkylation is observed *in vitro*, or if it is extremely slow, it may be concluded that the compound lacks significant alkylating capacity. It should be noted that this conclusion only applies to the tested compound itself; if the effective alkylating agent is formed during the metabolism of the compound studied, the NBP assay is useless unless it is carried out in simulated metabolic conditions. This absence of reactivity with NBP has been correlated with the lack of mutagenicity (e.g., neopentyl bromide and pentaerythrityl tetrachloride[55] or DDNU-oxide, a presumed metabolite of DDT).[42] It has also helped to understand the low alkylating potential attributed to weak electrophiles such as sorbic acid[9] and sorbates,[8] and acrylamide[12] and other $\alpha-\beta$ unsaturated carbonyls. All these compounds are stable in aqueous solution for as long as the experiment lasts, and they react very slowly, if at all, with NBP. This low reactivity is in agreement with the low or null *in vivo* activity of these compounds.

Occasionally, the assumption that the hydrolysis reaction can be neglected is quite arbitrary. For instance, Hooberman et al. found a good correlation between mutagenicity and the NBP alkylation rate in a study of seventeen propylene oxides. They attributed some of the deviations to epoxide decomposition, which they failed to include.[37] Also failing to acknowledge the hydrolysis reaction, Hemminki et al. compared the alkylation of DNA, deoxyguanosine, and NBP by linear epoxides. A better correlation was found for biological models, which was attributed to the fact that the NBP test was carried out under harsh, nonbiomimetic conditions.[34]

Styrene oxide derivatives are possibly the oxiranes most studied. For instance, the alkylation rate of styrene oxide derivatives, once again neglecting hydrolysis and variations in molar absorptivity, has been used to discuss the role of the site of nucleophilic attack on mutagenicity.[44]

Many authors have reported solid relationships between kinetic parameters and biological effects. For instance, a quantitative relationship was found between the alkylation rate and the ability to induce point mutations, mitotic gene conversion, and recombination in a diploid strain (D7) of *Saccharomyces cerevisiae* for 3,4-epoxycyclohexene and some analogues.[47] Also, the LC_{50} values of different epoxy compounds were correctly predicted using quantitative structure activity relationship (Q-SAR) based on the alkylation rate constant and hydrophobicity.[33] *N*-Alkyl-*N*-nitrosoureas decompose in water to form diazonium ions, the effective alkylating agents. The benzenediazonium ion reacts with NBP about 20,000-fold more slowly than methyldiazonium, which is consistent with the fact that the methyldiazonium ion shows higher carcinogenicity in animal tests.[197]

Mechanistic conclusions have also been obtained from this approach to the kinetic NBP assay. The relative hydrolysis and alkylation rates of *N*-diazoacetyl derivatives of amino acids suggest that degradation is a prerequisite for alkylation.[81] Peterson et al. concluded, partly from the alkylation and hydrolysis rates of some cysteinyl and homocysteinyl analogues of *S*-(2-haloethyl)glutathione (a carcinogenic metabolic product of ethylene dibromide), that the ethylene dibromide−glutathione conjugate forms an episulfonium ion prior to reaction with DNA guanyl residues.[198] Meier et al. studied the formation of alkylating agents in the nitrosation of some amino acids (aspartic acid, aspartame, and glycine ethylester),[137] and García-Santos et al. demonstrated that the alkylating species resulting from the nitrosation of amino acids with an -NH_2 group are the corresponding lactones.[199,200]

The use of the Initial Rate Method[201] (IRM) allows researchers to neglect both concurrent hydrolysis and subsequent adduct decomposition, even in cases where these are quite rapid, as long as the extent of the reaction remains low (under 5%). Thus, it is a very useful approach for the calculation of alkylation rate constants. However, knowledge of the adduct absorption coefficient is essential to calculate the alkylation rate constants.

$$r_o = k_{alk}[\mathrm{AA}]_o[\mathrm{NBP}]_o = \frac{1}{\varepsilon l}\frac{dA}{dt} \quad (7)$$

As in nonkinetic approaches, very often the same molar absorption coefficient is used for all the compounds in a study, although this may afford misleading results.

The kinetic NBP test has been applied most often to epoxides, and it is thus unsurprising that most examples of the application of the IRM, (and most usually the concomitant approximation regarding the molar extinction coefficients) are found in the study of alkylation by oxiranes. A good correlation has been found between the alkylation rate and mutagenicity of trichloropropylene oxide, epichlorohydrin, styrene oxide, glycidol, and propylene oxide;[36] 4-vinylcyclohexene metabolites;[48] and oxirane and vinyl oxirane.[56]

Allyl compounds have also received attention. Thus, Schiffmann et al. found a correlation between unscheduled DNA synthesis, mutagenicity in the Ames test, and the alkylation of NBP,[180] and Eder et al. found mutagenicity−alkylation rate correlations for six allylic alkylating agents.[54] The latter authors concluded that the determination of kinetic data and activation energies does not add significantly to the standard NBP test for the prediction of mutagenic potentials of the group of compounds under test. Indeed, the use of a kinetic approach without a mechanistic interpretation of the results means that the kinetic data are of little use.

Other systems for which reasonable rate−activity relationships have been found include organophosphate impurities of malathion;[67] pyrrolizidine alkaloid pyrroles[202] and their metabolites;[203] acridine-linked aniline mustards,[24] whose alkylation rate constant also correlates well with the ^{15}N

NMR shifts;[204] and quaternary ammonium derivatives of chlorambucil and melphalan.[205]

4.2.2.2. Competing Hydrolysis. Since alkylating agents are potent electrophiles, they often undergo hydrolysis in aqueous media, and hence, a parallel reaction must be considered (Scheme 3). The rate equation in this case is

$$[AD]_t = [AA]_o \frac{k_{alk}[NBP]}{k_{alk}[NBP] + k_{hyd}[H_2O]} \times (1 - e^{-(k_{alk}[NBP]+k_{hyd}[H_2O])t}) \quad (8)$$

Therefore, the experimentally observed rate constant is the sum of both the pseudofirst-order hydrolysis and the alkylation rate constants.

$$k_{obs} = k_{alk}[NBP] + k_{hyd}[H_2O] \quad (9)$$

From a kinetic point of view, selectivity toward NBP or alkylating efficiency can be expressed as the quotient between the alkylation rate constant and the hydrolysis constant as follows:

$$S_{NBP} = \frac{k_{alk}}{k_{hyd}} \quad (10)$$

The ratio between the concentrations of the alkylation and hydrolysis products, even for an S_N1-reacting agent, can be considered a *selectivity factor*, not necessarily derived from measurable kinetic rate constants.

Another useful parameter, which depends on the concentration of NBP, is the fraction of the alkylating agent that finally forms the adduct (f):

$$f = \frac{[AD]}{[AA]_o} = \frac{k_{alk}[NBP]}{k_{alk}[NBP] + k_{hyd}[H_2O]} \quad (11)$$

S_{NBP} and f are closely related since

$$\frac{1}{f} = 1 + \frac{1}{S_{NBP}} \frac{[H_2O]}{[NBP]} \quad (12)$$

Kawazoe et al. synthesized some *N*-Alkyl-4-(*p*-nitrobenzyl)-pyridonium salts and, making use of their absorption coefficients, calculated the S_{NBP} values for a number of S_N1 and S_N2 alkylating agents. They found that $\log(S_{NBP})$ ranged from 4.3 to 0.7 for some alkyl halides and were as low as −1 for some nitrosoureas and nitrosoguanidines.[101] The intermediate−low values indicate a large amount of competing hydrolysis (diethylsulfate, ethylmethanesulfonate, pentylmethanesulfonate...), whereas the very low values obtained for some compounds suggest that they act through S_N1 mechanisms.

Ninomiya et al. also calculated the S_{NBP} values for a number of alkyl sulfonates ($1.0 < \log(S_{NBP}) < 4.3$). They observed that the chemoselectivity depended strongly on the nature of the alkoxy group: the bulkier the substituent (and hence the larger the S_N1-character), the smaller the selectivity. The order of selectivity toward NBP for the compounds studied is therefore methyl > ethyl > isopropyl. The nature of the leaving group ($R_1SO_3^-$) is of less importance, although electron-deficient sulfonic groups tend to decrease NBP selectivity.[100] In terms of the alkylation rate constant, the order of reactivity is methyl > isopropyl > ethyl; the increase in the reactivity of the isopropyl group with respect to the ethyl group can be attributed to the S_N1 reaction.

Kagn and Spears characterized the reactivity of some selenium mustards by studying their alkylation rate and competitive hydrolysis, finding a reactivity very similar to that of nitrogen mustards, and pointed out their possible use as drugs.[206]

The values of both S_{NBP} and f have been used to correlate reactivity against NBP and carcinogenicity/mutagenicity. Thus, it has been observed that the doses required to equate the carcinogenic effects of β-propiolactone (BPL) and β-butyrolactone (BBL), which are considered as *possibly carcinogenic to humans* (IARC 2A), are well correlated with both their alkylation rate constants and their S_{NBP} values (Table 6).[7]

Table 6. Correlation of Tumorigenicity and NBP Alkylation Kinetics for β-Lactones[a]

	in vitro NBP alkylation		*in vivo* tumorigenicity			
			mice		rats	
lactone	k_{alk} (10 M^{-1} min^{-1})	S_{NBP}	dose (mg)	tumors/animals	dose (mg)	tumors/animals
BPL	8.2 ± 0.3	5000	4	13/20	0.73	18/30
BBL	0.78 ± 0.04	3200	100	9/20	10	18/30

[a]Taken from ref 7. T = 37.5 °C; 7:3 water/dioxane medium. $[NBP]_o = 2 \times 10^{-2}$ M.

The competing hydrolysis reaction is significant, but not major. Similar results were obtained by Hemminki, although this author neglected the parallel hydrolysis reaction.[35]

As regards *N*-nitrosoureas, which decompose to form alkylating diazonium ions, it has been observed that their f and S_{NBP} values correlate with their tumorigenicity in test animals.[15]

1,4-Dinitro-2-methylpyrrole (NMP), a product of the reaction between nitrite and sorbic acid, both of which are common food additives, is a proven mutagen. A joint experimental/*in silico* study of the NBP alkylation mechanism revealed that the effective alkylating agent is 5-methyl-3-nitro-2*H*-pyrrol-2-ol, a decomposition product formed after nucleophilic attack of a hydroxide ion, the loss of nitrite, and subsequent isomerization. The somewhat low f value obtained (0.07; S_{NBP} = 155 at 37.5 °C in 7:3 water/dioxane medium; $[NBP]_o$ = 0.02 M) is coherent with the low mutagenicity of the compound.[11]

4.2.2.3. Adduct Decomposition. In more complex cases, the adducts formed are unstable and undergo hydrolysis in the reaction medium. This reactivity explains the disappearance of color for some cromophores and their increased stability in nonaqueous media. In general, this decomposition reaction has been observed to undergo general base catalysis by hydroxide, acetate, phosphate, and even NBP itself.[141,207] In these cases, the alkylation efficiency does not include all possible effects, and a new parameter was introduced to take into account the decomposition of the NBP-AA adduct: adduct life (AL).

$$AL = \frac{\int_0^\infty [AD]dt}{[AA]_o} = \int_0^\infty \frac{k_{alk}[NBP]}{k_{dec} - k_{alk}[NBP] - k_{hyd}[H_2O]} \times (e^{-(k_{alk}[NBP]+k_{hyd}[H_2O])t} - e^{-k_{dec}t}) \quad (13)$$

$$AL = \frac{k_{alk}[NBP]}{(k_{alk}[NBP] + k_{hyd}[H_2O])k_{dec}} \quad (14)$$

AL is defined as the area under the kinetic profile of the reaction per unit of alkylating agent. Since it includes all possible effectors (the alkylation rate, the rate of the competing hydrolysis, and the adduct stability), this parameter gives an idea of the *cumulative* effect of an alkylating agent, combining the concentration and time factors. A higher AL implies a combination of more adduct being formed and this having a longer life and thus more time to exert its biological effect.

Since AL depends directly on *f*, a greater selectivity for NBP implies a higher concentration of adduct and hence a larger cumulative effect over time.

$$AL = \frac{f}{k_{dec}} \quad (15)$$

In addition, the faster the adduct decomposition, the lower the AL (Table 7).

Table 7. Alkylating Agents That Form Unstable Adducts

molecule	log S_{NBP}	*f*	AL (min)
ethylnitrolic acid[10]	2.5	0.13	7.3
p-nitrostyrene oxide[6]	4.0	0.82	3300
diketene	5.7	1.0	1.2

T = 37.5 °C; 7:3 water/dioxane medium. $[NBP]_o = 2 \times 10^{-2}$ M.

Ethylnitrolic acid[10,208] (ENA), a product of the reaction between nitrite and sorbic acid, is a proven mutagen. ENA decomposes in aqueous medium, which, together with the presence of an induction period in the reaction between ENA and NBP, suggests that the active alkylating agent is a decomposition product of ENA. In aqueous media, ethylnitrolic acid is transformed into acetonitrile oxide, the active species that alkylates NBP. The adduct thus formed is rather short-lived, and both the low *f* and AL values are consistent with its low mutagenicity (Table 7).

The alkylating potential of α-angelicalactone (AAL), a γ-lactone with chemopreventive activity, has also been investigated on the basis of its alkylation rate and AL.[13] The extremely high value of k_{alk} (the alkylation reaction lasting less than a second) suggests that α-angelicalactone might react rapidly with the most exposed positions, protecting them from other alkylating agents. The short life of the AAL-DNA adducts would then prevent α-angelicalactone from causing permanent DNA damage.

Diketene (DIK), a β-lactone, reacts rapidly with NBP by acyl-oxygen cleavage. The alkylation reaction is much faster than the hydrolysis of DIK,[209] and thus an *f* value close to unity is obtained. However, the adduct formed is an aromatic amide, rather than the pyridonium cation afforded by lactones that undergo alkyl-cleavage such as BPL and BBL. This amide is very labile and undergoes rapid hydrolysis in the reaction conditions. The low value obtained for AL (Table 7) is in keeping with the lack of biological effects: if formed, the DNA adducts are expected to undergo hydrolysis within few minutes, which prevents them from having *in vivo* effects.[14]

p-Nitrostyrene oxide (*p*NSO), a mutagenic compound used as a model for epoxide hydrolase activity due to its slow spontaneous hydrolysis, has also been investigated using the NBP test. Although styrene oxides generally react through both the α and β carbons of the oxirane ring, α adduct formation is negligible for *p*NSO. The hydrolysis reaction of the epoxide competes with the formation of the unstable β adduct, whose decomposition is also relevant.[6] The high *f* (0.82) and low AL (3300 min) values indicate that it is a strong alkylating agent with low alkylating effectiveness. This is in accordance with the lower mutagenicity of *p*NSO in comparison with BPL, which has a similar *f* value but forms a stable adduct.

5. CONCLUSIONS

The first application of 4-(*p*-nitrobenzyl)pyridine to detect alkylating agents was in 1925.[1] Half a century later, Francis Crick wrote, "DNA is such an important molecule that it is almost impossible to learn too much about it."[210] In 2009, Kent S. Gates wrote, "Characterization of the chemical reactions of endogenous cellular chemicals, anticancer drugs, and mutagens with cellular DNA is important because the biological responses engendered by any given DNA-damaging agent are ultimately determined by the chemical structure of the damaged DNA."[2]

Since (i) a large number of mechanisms by which environmental molecules cause DNA damage are alkylation processes; (ii) several antitumor drugs are also alkylating, carcinogenic, and mutagenic agents; and (iii) NBP is known to react with strong and weak alkylating agents, and much insight into alkylation mechanisms *in vivo* can be gained from the study *in vitro*, the NBP assay can be viewed as an easy and efficient method for the assessment of the genetic risks of the vast majority of current, as well as emergent, alkylating agents.

From this perspective, we believe the main conclusion to be drawn from the studies reviewed here is that since 1925 the NBP assay has evolved from being a qualitative, analytical tool to being a useful physicochemical method that not only allows the rules of chemical reactivity that govern electrophilicity and nucleophilicity to be applied to the reaction of DNA with alkylating agents but also helps to understand some significant relationships between the structure of many alkylation substrates (including DNA) and their chemical and biological responses. Advances in this area have the potential to yield both fundamental and practical advances in chemistry, biology, predictive toxicology, and anticancer drug development.

■ NOTE ADDED IN PROOF

Two recent examples for the application of the NBP Method to the kinetic study of alkylation mechanisms are as follows: Céspedes-Camacho, I. F., Manso, J. A., González Jiménez, M., Calle, E., and Casado, J. (2012) The reactivity of vinyl compounds as alkylating agents. *Monatsh. Chem. 143,* 723−727.

Arenas-Valgañón, J., Gómez-Bombarelli, R., González-Pérez, M., González-Jiménez, M., Calle, E., and Casado, J. (2012) Taurine-nitrite interaction as a precursor of alkylation mechanisms. *Food Chem. 134,* 986−991.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (+34) 923 294486. Fax: (+34) 923 294574. E-mail: jucali@usal.es

Funding

We thank the Spanish Ministerio de Ciencia e Innovación and the European Regional Development Fund (Project CTQ2010-18999) for supporting the research reported in this review. R.G.B. and M.G.P. thank the Spanish Ministerio de Educación (FPU: AP2006-1976) and the Junta de Castilla y León-FEDER Funds, respectively, for Ph.D. fellowships.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. P.F. Hollenberg and referees of the manuscript for their valuable comments. Thanks are also given to Jorge Arenas-Valgañón for his revision of the manuscript.

■ ABBREVIATIONS

AD, adduct; AL, adduct life; AA, alkylating agent; AAL, α-angelicalactone; HN2, bis(2-chloroethyl)methylamine; nor-HN2, bis(2-chloroethyl)amine; BBL, β-butyrolactone; CAS, Chemical Activation System; DDT, *p,p*-1,1-dichloro-2,2-bis (chlorophenyl)-ethane; DDD, *p,p*-1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane; DDE, *p,p*-1,1-dichloro-2,2-bis(*p*chlorophenyl) ethylene; DDMU, *p,p*-1,1-dichloro-2,2-bis(*p*-chlorophenyl)-2-chloroethylene; DDNU, *p,p*-1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene; DIK, diketene; ENA, ethylnitrolic acid; IRM, Initial Rate Method; ppb, parts per billion; NBP, 4-(*p*-nitrobenzyl)pyridine; *p*NSO, *p*-nitrostyrene oxide; BPL, β-propiolactone

■ REFERENCES

(1) Lawley, P. (1976) Carcinogenesis by Alkylating Agents, in *Chemical Carcinogens* (Searle, C. E., Ed.) pp 83−244, American Chemical Society, Washington, DC.

(2) Gates, K. S. (2009) An overview of chemical processes that damage cellular DNA: spontaneous hydrolysis, alkylation, and reactions with radicals. *Chem. Res. Toxicol. 22*, 1747−1760.

(3) Epstein, J., Rosenthal, R. W., and Ess, R. J. (1955) Use of g-(4-nitrobenzyl)pyridine as analytical reagent for ethylenimines and alkylating agents. *Anal. Chem. 27*, 1435−1439.

(4) Kim, J. H., and Thomas, J. J. (1992) Use of 4-(nitrobenzyl)-pyridine (4-NBP) to test mutagenic potential of slow-reacting epoxides, their corresponding olefins, and other alkylating-agents. *Bull. Environ. Contam. Toxicol. 49*, 879−885.

(5) Gómez-Bombarelli, R., González-Pérez, M., Arenas-Valgañón, J., Céspedes-Camacho, I. F., Calle, E., and Casado, J. (2011) DNA-damaging disinfection byproducts: Alkylation mechanism of mutagenic mucohalic acids. *Environ. Sci. Technol. 45*, 9009−9016.

(6) González-Pérez, M., Gómez-Bombarelli, R., Pérez-Prior, M. T., Manso, J. A., Céspedes-Camacho, I. F., Calle, E., and Casado, J. (2011) Reactivity of p-nitrostyrene oxide as an alkylating agent. A kinetic approach to biomimetic conditions. *Org. Biomol. Chem. 9*, 7016−7022.

(7) Manso, J. A., Pérez-Prior, M. T., García-Santos, M. P., Calle, E., and Casado, J. (2005) A kinetic approach to the alkylating potential of carcinogenic lactones. *Chem. Res. Toxicol. 18*, 1161−1166.

(8) Pérez-Prior, M. T., Manso, J. A., García-Santos, M. P., Calle, E., and Casado, J. (2005) Alkylating potential of potassium sorbate. *J. Agric. Food Chem. 53*, 10244−10247.

(9) Pérez-Prior, M. T., Manso, J. A., García-Santos, M. P., Calle, E., and Casado, J. (2008) Sorbic acid as an alkylating agent. *J. Solution Chem. 37*, 459−466.

(10) Pérez-Prior, M. T., Gómez-Bombarelli, R., González-Pérez, M., Manso, J. A., García-Santos, M. P., Calle, E., and Casado, J. (2009) Sorbate-nitrite interactions: acetonitrile oxide as an alkylating agent. *Chem. Res. Toxicol. 22*, 1320−1324.

(11) Pérez-Prior, M. T., Gómez-Bombarelli, R., González-Pérez, M., Manso, J. A., García-Santos, M. P., Calle, E., and Casado, J. (2010) Reactivity of the mutagen 1,4-dinitro-2-methylpyrrole as an alkylating agent. *J. Org. Chem. 75*, 1444−1449.

(12) Céspedes, I. F., Manso, J. A., Pérez-Prior, M. T., Gómez-Bombarelli, R., González-Pérez, M., Calle, E., and Casado, J. (2010) Reactivity of acrylamide as an alkylating agent: A kinetic approach. *J. Phys. Org. Chem. 23*, 171−175.

(13) Fernández-Rodríguez, E., Manso, J. A., Pérez-Prior, M. T., García-Santos, M. P., Calle, E., and Casado, J. (2007) The unusual ability of alpha-angelicalactone to form adducts: A kinetic approach. *Int. J. Chem. Kinet. 39*, 591−594.

(14) Gómez-Bombarelli, R., González-Pérez, M., Teresa Pérez-Prior, M., Manso, J. A., Calle, E., and Casado, J. (2008) Chemical reactivity and biological activity of diketene. *Chem. Res. Toxicol. 21*, 1964−1969.

(15) Manso, J. A., Pérez-Prior, M. T., García-Santos, M. P., Calle, E., and Casado, J. (2008) Steric effect in alkylation reactions by N-alkyl-N-nitrosoureas: a kinetic approach. *J. Phys. Org. Chem. 21*, 932−938.

(16) Gates, K. S. (1999) Covalent Modification of DNA by Natural Products, in *Comprehensive Natural Products Chemistry* (Kool, E. T., Ed.) pp 491−552, Pergamon, New York.

(17) Wolkenberg, S. E., and Boger, D. L. (2002) Mechanisms of *in situ* activation for DNA-targeting antitumor agents. *Chem. Rev. 102*, 2477−2495.

(18) Hurley, L. H. (2002) DNA and its associated processes as targets for cancer therapy. *Nat. Rev. Cancer 2*, 188−200.

(19) Sobol, Z., Engel, M. E., Rubitski, E., Ku, W. W., Aubrecht, J., and Schiestl, R. H. (2007) Genotoxicity profiles of common alkyl halides and esters with alkylating activity. *Mutat. Res. 633*, 80−94.

(20) Decker, H. (1905) Ueber die Beziehungen des doppelt gebundenen Kohlenstoffs zum Stickstoff, Sauerstoff und Schwefel. *Ber. Dtsch. Chem. Ges. 38*, 2493−2511.

(21) Koenigs, E., Kohler, K., and Blindow, K. (1925) Regarding pryidone methide. *Ber. Dtsch. Chem. Ges. 58*, 933−940.

(22) Agarwal, S. C., Van Duuren, B. L., and Kneip, T. J. (1979) Detection of epoxides with 4-(p-nitrobenzyl) pyridine. *Bull. Environ. Contam. Toxicol. 23*, 825−829.

(23) Bardos, T. J., Datta-Gupta, N., Hebborn, P., and Triggle, D. J. (1965) A study of comparative chemical and biological activities of alkylating agents. *J. Med. Chem. 8*, 167−174.

(24) Gourdie, T. A., Valu, K. K., Gravatt, G. L., Boritzki, T. J., Baguley, B. C., Wakelin, L. P. G, Wilson, W. R., Woodgate, P. D., and Denny, W. A. (1990) DNA-directed alkylating agents. 1. Structure-activity relationships for acridine-linked aniline mustards: consequences of varying the reactivity of the mustard. *J. Med. Chem. 33*, 1177−1186.

(25) Perlman, M. E., and Bardos, T. J. (1988) Synthesis, molecular symmetry, and chemical-reactivity of C-aryl-substituted phosphoraziridines. *J. Org. Chem. 53*, 1761−1767.

(26) Sims, P. (1973) Epoxy derivatives of aromatic polycyclic-hydrocarbons: preparation and metabolism of epoxides related to 7,12-dimethylbenz[alpha]anthracene. *Biochem. J. 131*, 405−413.

(27) Swaisland, A. J., Grover, P. L., and Sims, P. (1973) Some properties of "K-region" epoxides of polycyclic aromatic hydrocarbons. *Biochem. Pharmacol. 22*, 1547−1556.

(28) Van Duuren, B. L., Melchionne, S., Seidman, I., and Pereira, M. A. (1986) Chronic bioassays of chlorinated humic acids in B6c3f1 mice. *Environ. Health Perspect. 69*, 109−117.

(29) Wilman, D. E. V., Palmer, B. D., and Denny, W. A. (1995) Application of 15N nuclear magnetic resonance spectroscopy to the determination of the stability of aryl nitrogen mustards. *J. Med. Chem. 38*, 2256−2258.

(30) Banerjee, R., Rachid, Z., McNamee, J., and Jean-Claude, B. J. (2003) Synthesis of a prodrug designed to release multiple inhibitors of the epidermal growth factor receptor tyrosine kinase and an alkylating agent: A novel tumor targeting concept. *J. Med. Chem. 46*, 5546−5551.

(31) Barbin, A., Bresil, H., Croisy, A., Jacquignon, P., Malaveille, C., Montesano, R., and Bartsch, H. (1975) Liver-microsome-mediated formation of alkylating-agents from vinyl bromide and vinyl-chloride. *Biochem. Biophys. Res. Commun. 67*, 596−603.

(32) Bartsch, H., Terracini, B., Malaveille, C., Tomatis, L., Wahrendorf, J., Brun, G., and Dodet, B. (1983) Quantitative comparison of carcinogenicity, mutagenicity and electrophilicity of 10 direct-acting alkylating agents and of the initial O6: 7-alkylguanine ratio in DNA with carcinogenic potency in rodents. *Mutat. Res.-Fundam. Mol. Mech. Mutag. 110*, 181−219.

(33) Deneer, J. W., Sinnige, T. L., Seinen, W., and Hermens, J. L. M. (1988) A quantitative structure activity relationship for the acute

toxicity of some epoxy compounds to the guppy. *Aquat. Toxicol. 13*, 195−204.
(34) Hemminki, A., Vayrynen, T., and Hemminki, K. (1994) Reaction-kinetics of alkyl epoxides with DNA and other nucleophiles. *Chem.-Biol. Interact. 93*, 51−58.
(35) Hemminki, K. (1981) Reactions of β-propiolactone, β-butyrolactone and γ-butyrolactone with nucleic-acids. *Chem.-Biol. Interact. 34*, 323−331.
(36) Hemminki, K., and Falck, K. (1979) Correlation of mutagenicity and 4-(*p*-nitrobenzyl)-pyridine alkylation by epoxides. *Toxicol. Lett. 4*, 103−106.
(37) Hooberman, B. H., Chakraborty, P. K., and Sinsheimer, J. E. (1993) Quantitative structure-activity relationships for the mutagenicity of propylene oxides with Salmonella. *Mutat. Res.-Genet. Toxicol. Environ. Mutag. 299*, 85−93.
(38) Malaveille, C., Brun, G., Kolar, G., and Bartsch, H. (1982) Mutagenic and alkylating activities of 3-methyl-1-phenyltriazenes and their possible role as carcinogenic metabolites of the parent dimethyl compounds. *Cancer Res. 42*, 1446−1453.
(39) Metzler, M. (1978) Synthesis of diethylstilbestrol metabolites - omega-hydroxy-dienestrol and derivatives. *Tetrahedron 34*, 3113−3117.
(40) Nelis, H. J. C. F., Airy, S. C., and Sinsheimer, J. E. (1982) Comparison of the alkylation of nicotinamide and 4-(p-nitrobenzyl)-pyridine for the determination of aliphatic epoxides. *Anal. Chem. 54*, 213−216.
(41) Padgette, S. R., Wimalasena, K., Herman, H. H., Sirimanne, S. R., and May, S. W. (1985) Olefin oxygenation and N-dealkylation by dopamine beta-monooxygenase: catalysis and mechanism-based inhibition. *Biochemistry 24*, 5826−5839.
(42) Planche, G., Croisy, A., Malaveille, C., Tomatis, L., and Bartsch, H. (1979) Metabolic and mutagenicity studies on DDT and 15 derivatives. Detection of 1.1-bis(*p*-chlorophenyl)-2,2-dichloroethane and bis(*p*-chlorophenyl)-2,2,2-trichloroethyl acetate (kelthane acetate) as mutagens in *Salmonella typhimurium* and of 1.1-bis(*p*-chlorophenyl) ethylene oxide, a likely metabolite, as an alkylating agent. *Chem.-Biol. Interact. 25*, 157−175.
(43) Robertson, K. A. (1982) Alkylation of N-2 in deoxyguanosine by dehydroretronecine, a carcinogenic metabolite of the pyrrolizidine alkaloid monocrotaline. *Cancer Res. 42*, 8−14.
(44) Rosman, L. B., Beylin, V. G., Gaddamidi, V., Hooberman, B. H., and Sinsheimer, J. E. (1986) Mutagenicity of *para*-substituted α-methylstyrene oxide derivatives with *Salmonella*. *Mutat. Res. 171*, 63−70.
(45) Sinsheimer, J. E., Chakraborty, P. K., Messerly, E. A., and Gaddamidi, V. (1989) Mutagenicity of oxaspiro compounds with *Salmonella*. *Mutat. Res.-Genet. Toxicol. Environ. Mutag. 224*, 171−175.
(46) Thier, R., Muller, M., Taylor, J. B., Pemble, S. E., Ketterer, B., and Guengerich, F. P. (1995) Enhancement of bacterial mutagenicity of bifunctional alkylating agents by expression of mammalian glutathione S-transferase. *Chem. Res. Toxicol. 8*, 465−472.
(47) Turchi, G., Bauer, C., Bronzetti, G., Citti, L., Corsi, C., Fassina, G. F., Gervasi, P. G., Lippi, A., Nieri, R., Abbondandolo, A., Berti, G., and Mastrorilli, E. (1983) Mutagenicity of 3 structurally related epoxides, with defined stereochemical configuration, in Saccharomyces-Cerevisiae and in V79 Chinese-hamster cells. *Mutat. Res. 117*, 213−224.
(48) Turchi, G., Bonatti, S., Citti, L., Gervasi, P. G., Abbondandolo, A., and Presciuttini, S. (1981) Alkylating properties and genetic-activity of 4-vinylcyclohexene metabolites and structurally related epoxides. *Mutat. Res. 83*, 419−430.
(49) Sawicki, E., Bender, D. F., Houser, T. R., Wilson, R. M., and Meeker, J. E. (1963) Five new methods for the spectrophotometric determination of alkylating agents including some extremely sensitive autocatalytic methods. Application to air pollution. *Anal. Chem. 35*, 1479−1486.
(50) Connors, T. A., Elson, L. A., and Leese, C. L. (1964) The effect of glucose pretreatment on the anti-tumour action of Mannitol Myleran. *Biochem. Pharmacol. 13*, 963−968.
(51) Eder, E., Neudecker, T., Lutz, D., and Henschler, D. (1980) Mutagenic potential of allyl and allylic compounds: Structure-activity relationship as determined by alkylating and direct in vitro mutagenic properties. *Biochem. Pharmacol. 29*, 993−998.
(52) Neudecker, T., Lutz, D., Eder, E., and Henschler, D. (1980) Structure-activity relationship in halogen and alkyl substituted allyl and allylic compounds: Correlation of alkylating and mutagenic properties. *Biochem. Pharmacol. 29*, 2611−2617.
(53) Raïkova, E., Grueva, D., and Golovinsky, E. (1981) In vivo investigation of some metabolic changes of 3-[bis-(2-chlorethyl)-carbohydrazide]-2,2,5,5-tetramethylpyroline-1-oxyl by the NBP-reaction and ESR method. *Biochem. Pharmacol. 30*, 289−291.
(54) Eder, E., Neudecker, T., Lutz, D., and Henschler, D. (1982) Correlation of akylating and mtagenic activities of allyl and allylic compounds: Standard alkylation test vs kinetic investigation. *Chem.-Biol. Interact. 38*, 303−315.
(55) Ashby, J., Callander, R. D., and Gilman, D. (1984) Lack of mutagenicity to *S. typhimurium* of neopentyl bromide and pentaerythrityl tetrachloride: relation to chemical structure. *Mutat. Res. Lett. 140*, 71−74.
(56) Schmiedel, G., Filser, J. G., and Bolt, H. M. (1983) Rat liver microsomal transformation of ethene to oxirane in vitro. *Toxicol. Lett. 19*, 293−297.
(57) Zoetemelk, C. E. M., Mohn, G. R., Vandergen, A., and Breimer, D. D. (1987) 1,2-Dibromo compounds: Their mutagenicity in *Salmonella* strains differing in glutathione content and their alkylating potential. *Biochem. Pharmacol. 36*, 1829−1835.
(58) Hill, B. T., and Harrap, K. R. (1972) Modification of the alkylating ability of cyclophosphamide by fresh plasma. *Chem.-Biol. Interact. 5*, 117−125.
(59) Jablonkai, I. (2003) Alkylating reactivity and herbicidal activity of chloroacetamides. *Pest Manage. Sci. 59*, 443−450.
(60) Juma, F. D., Rogers, H. J., and Trounce, J. R. (1979) Pharmacokinetics of cyclophosphamide and alkylating activity in man after intravenous and oral-administration. *Br. J. Clin. Pharmacol. 8*, 209−217.
(61) Juma, F. D., Rogers, H. J., and Trounce, J. R. (1980) The pharmacokinetics of cyclophosphamide, phosphoramide mustard and nor-nitrogen mustard studied by gas-chromatography in patients receiving cyclophosphamide therapy. *Br. J. Clin. Pharmacol. 10*, 327−335.
(62) Magata, Y., Lang, L., Kiesewetter, D. O., Jagoda, E. M., Channing, M. A., and Eckelman, W. C. (2000) Biologically stable [^{18}F]-labeled benzylfluoride derivatives. *Nucl. Med. Biol. 27*, 163−168.
(63) Preussmann, R., Schneider, H., and Epple, F. (1969) Untersuchungen zum Nachweis alkylierender Agentien. *Arzneim.-Forsch. 19*, 1059−1073.
(64) Braun, R., Dittmar, W., Hefter, B., and Weber, K. (1981) On interactions of cytostatic benzylidine hydrazines with SH-groups. *Chem.-Biol. Interact. 35*, 229−239.
(65) Miyahara, M., Miyahara, M., Kamiya, S., and Maekawa, A. (1981) Anti-tumor activity of 3-nitroso-2-oxazolidones. *Chem. Pharm. Bull. 29*, 2366−2369.
(66) Budzisz, E., Nawrot, E., and Malecka, M. (2001) Synthesis, antimicrobial, and alkylating properties of 3-phosphonic derivatives of chromone. *Arch. Pharm. 334*, 381−387.
(67) Imamura, T., and Talcott, R. E. (1985) Mutagenic and alkylating activities of organophosphate impurities of commercial malathion. *Mutat. Res.-Genet. Toxicol. Environ. Mutag. 155*, 1−6.
(68) Kalinowska-Lis, U., Szmigiero, L., Studzian, K., and Ochocki, J. (2009) Cytotoxic activity and chemical reactivity of cis-platinum(II) and trans-palladium(II) complexes with diethyl (pyridinylmethyl)-phosphates. *Eur. J. Med. Chem. 44*, 660−664.
(69) Katzenellenbogen, J. A., McGorrin, R. J., Tatee, T., Kempton, R. J., Carlson, K. E., and Kinder, D. H. (1981) Chemically reactive estrogens: synthesis and estrogen receptor interactions of hexestrol ether derivatives and 4-substituted deoxyhexestrol derivatives bearing alkylating functions. *J. Med. Chem. 24*, 435−450.

(70) Nawrot-Modranka, J., Nawrot, E., and Graczyk, J. (2006) In vivo antitumor, in vitro antibacterial activity and alkylating properties of phosphorohydrazine derivatives of coumarin and chromone. *Eur. J. Med. Chem. 41*, 1301−1309.
(71) Bagley, C. M., Jr., Bostick, F. W., and DeVita, V. T., Jr. (1973) Clinical pharmacology of cyclophosphamide. *Cancer Res. 33*, 226−233.
(72) Chmielewicz, Z. F., Fiel, R. J., Bardos, T. J., and Ambrus, J. L. (1967) Alterations of some macromolecular and biochemical properties of calf thymus DNA caused by dual antagonists and nitrogen mustard. *Cancer Res. 27*, 1248−1257.
(73) Friedman, O. M., and Boger, E. (1961) Colorimetric estimation of nitrogen mustards in aqueous media. Hydrolytic behavior of bis-(β-chloroethyl)amine, nor HN2. *Anal. Chem. 33*, 906−910.
(74) Truhaut, R., Delacoux, E., Brule, G., and Bohuon, C. (1963) Dosage des agents alcoylants dans les milieux biologiques Méthode utilisant la réaction colorée avec la γ-(nitro-4-benzyl)-pyridine en milieu alcalin. *Clin. Chim. Acta 8*, 235−245.
(75) Hsieh, K. H., and Marshall, G. R. (1981) Alkylating angiotensin-II analogs: synthesis, analysis, and biological-activity of angiotensin II analogs containing the nitrogen-mustard melphalan in position-8. *J. Med. Chem. 24*, 1304−1310.
(76) Acosta, D., and Mitchell, D. B. (1981) Metabolic-activation and cyto-toxicity of cyclophosphamide in primary cultures of postnatal rat hepatocytes. *Biochem. Pharmacol. 30*, 3225−3230.
(77) Archer, M. C., and Eng, V. W. S. (1981) Quantitative-determination of carcinogens and mutagens as alkylating-agents following chemical activation. *Chem.-Biol. Interact. 33*, 207−214.
(78) Chan, K. K., Hong, P. S., Tutsch, K., and Trump, D. L. (1994) Clinical pharmacokinetics of cyclophosphamide and metabolites with and without SR-2508. *Cancer Res. 54*, 6421−6429.
(79) Fisher, G. R., Donis, J., and Gutiérrez, P. L. (1992) Reductive metabolism of diaziquone (AZQ) in the S9 fraction of MCF-7 cells: II. Enhancement of the alkylating activity of AZQ by NAD(P)H: quinone-acceptor oxidoreductase (DT-diaphorase). *Biochem. Pharmacol. 44*, 1625−1635.
(80) Hipkens, J. H., Struck, R. F., and Gurtoo, H. L. (1981) Role of aldehyde dehydrogenase in the metabolism-dependent biological activity of cyclophosphamide. *Cancer Res. 41*, 3571−3583.
(81) Parodi, S., Picca, M., Bolognesi, C., Cavanna, M., Carlo, P., Finollo, R., and Brambilla, G. (1977) Alkylation of [gamma]-(4-nitrobenzyl)pyridine by four N-diazoacetyl derivatives of amino acids. Lack of fine correlation with the unscheduled DNA synthesis induced in mouse cells "in vitro". *Pharmacol. Res. Commun. 9*, 621−631.
(82) Samanta, S., Pain, A., Ghosh, M., Dutta, S., and Sanyal, U. (2005) Evaluation of fluorenhymustine as a rationally designed novel anticancer agent. *Exp. Oncol. 27*, 279−285.
(83) Schasteen, C. S., and Reed, D. J. (1983) The hydrolysis and alkylation activities of S-(2-haloethyl)-L-cysteine analogs: evidence for extended half-life. *Toxicol. Appl. Pharmacol. 70*, 423−432.
(84) Schmid, F. A., Otter, G. M., and Mehta, B. M. (1985) Mutagenic and chemotherapeutic activity in L1210 leukemia of several monofunctional alkylating-agents. *Cancer Res. 45*, 40−44.
(85) Shyam, K., Cosby, L. A., and Sartorelli, A. C. (1986) 1,2-Bis(arylsulfonyl)hydrazines. 2. The influence of arenesulfonyl and aralkylsulfonyl substituents on antitumor and alkylating activity. *J. Med. Chem. 29*, 1323−1325.
(86) van Maanen, M. J., Tijhof, I. M., Damen, J. M. A., Versluis, C., van den Bosch, J. J. K., Heck, A. J. R., Rodenhuis, S., and Beijnen, J. H. (1999) A search for new metabolites of N,N′,N″-triethylenethiophosphoramide. *Cancer Res. 59*, 4720−4724.
(87) Wheeler, G. P., and Chumley, S. (1967) Alkylating activity of 1,3-bis(2-chloroethyl)-1-nitrosourea and related compounds. *J. Med. Chem. 10*, 259−261.
(88) Chakrabarty, S., Danels, Y. J., Long, B. H., Willson, J. K. V., and Brattain, M. G. (1986) Circumvention of deficient activation in mitomycin C-resistant human colonic-carcinoma cells by the mitomycin-C analog Bmy25282. *Cancer Res. 46*, 3456−3458.
(89) Kennedy, K. A., Mimnaugh, E. G., Trush, M. A., and Sinha, B. K. (1985) Effects of glutathione and ethylxanthate on mitomycin-C activation by isolated rat hepatic or EMT6 mouse mammary-tumor nuclei. *Cancer Res. 45*, 4071−4076.
(90) Kennedy, K. A., Sligar, S. G., Polomski, L., and Sartorelli, A. C. (1982) Metabolic-activation of mitomycin-C by liver-microsomes and nuclei. *Biochem. Pharmacol. 31*, 2011−2016.
(91) Marshall, R. S., Paterson, M. C., and Rauth, A. M. (1991) Studies on the mechanism of resistance to mitomycin-C and porfiromycin in a human cell strain derived from a cancer-prone individual. *Biochem. Pharmacol. 41*, 1351−1360.
(92) Lewis, L. D., Burton, L. C., Harper, P. G., and Rogers, H. J. (1992) Uroepithelial and nephrotubula toxicity in patients receiving ifosfamide/mesna: Measurement of urinary *N*-acetyl-β-D-glucosaminidase and β-2-microglobulin. *Eur. J. Cancer 28*, 1976−1981.
(93) Lewis, L. D., Fitzgerald, D. L., Harper, P. G., and Rogers, H. J. (1990) Fractionated ifosfamide therapy produces a time-dependent increase in ifosfamide metabolism. *Br. J. Clin. Pharmacol. 30*, 725−732.
(94) Lewis, L. D., Fitzgerald, D. L., Mohan, P., Thatcher, N., Harper, P. G., and Rogers, H. J. (1991) The pharmacokinetics of ifosfamide given as short and long intravenous infusions in cancer-patients. *Br. J. Clin. Pharmacol. 31*, 77−82.
(95) Aeschlimann, C., Kupfer, A., Schefer, H., and Cerny, T. (1998) Comparative pharmacokinetics of oral and intravenous ifosfamide/mesna methylene blue therapy. *Drug Metab. Dispos. 26*, 883−890.
(96) Colvin, M., Brundrett, R. B., Kan, M.-N. N., Jardine, I., and Fenselau, C. (1976) Alkylating properties of phosphoramide mustard. *Cancer Res. 36*, 1121−1126.
(97) Skibba, J. L., and Collins, F. G. (1980) Modification of the 4-(para-nitrobenzyl) pyridine assay for measurement of melphalan and mechlorethamine in plasma. *J. Pharmacol. Methods 4*, 155−163.
(98) Kang, S. I., and Spears, C. P. (1987) Linear free energy relationships and cytotoxicities of para-substituted 2-haloethyl aryl selenides and bis(2-chloroethyl) selenides. *J. Med. Chem. 30*, 597−602.
(99) Mochizuki, M., Michihiro, K., Shiomi, K., Itoh, K., Tange, Y., and Hizatate, S. (1993) Autooxidation of alkylhydrazones and mutagenicity of the resulting hydroperoxides. *Biol. Pharm. Bull. 16*, 96−98.
(100) Ninomiya, S., Kohda, K., and Kawazoe, Y. (1984) Studies on chemical carcinogens and mutagens. XXV. Chemoselectivity of alkyl sulfonates toward 4-(*p*-Nitrobenzyl)-pyridine (NBP) in phosphate buffer. *Chem. Pharm. Bull. 32*, 1326−1332.
(101) Kawazoe, Y., Tamura, N., and Yoshimura, T. (1982) Studies on chemical carcinogens. XXIII. A simple method for characterization of the alkylating ability of compounds by using 4-(*p*-Nitrobenzyl)-pyridine. *Chem. Pharm. Bull. 30*, 2077−2086.
(102) Cioli, D., Picamattoccia, L., Rosenberg, S., and Archer, S. (1985) Evidence for the mode of antischistosomal action of hycanthone. *Life Sci. 37*, 161−167.
(103) Cohen, B. E., Egorin, M. J., Nayar, M. S. B., and Gutiérrez, P. L. (1984) Effects of pH and temperature on the stability and decomposition of N,N′N″-triethylenethiophosphoramide in urine and buffer. *Cancer Res. 44*, 4312−4316.
(104) Egorin, M. J., and Snyder, S. W. (1990) Characterization of non-exchangeable radioactivity in L1210 cells incubated with [C-14] Thiotepa - Labeling of phosphatidylethanolamine. *Cancer Res. 50*, 4044−4049.
(105) Egorin, M. J., Snyder, S. W., Pan, S.-s., and Daly, C. (1989) Cellular transport and accumulation of thiotepa in murine, human, and avian cells. *Cancer Res. 49*, 5611−5617.
(106) El Masry, A. H., Braun, V. C., Nielsen, C. J., and Pratt, W. B. (1977) Synthesis and biological action of two gluco corticoid alkylating agents. *J. Med. Chem. 20*, 1134−1139.
(107) Harrach, B., Mirocha, C. J., Pathre, S. V., and Palyusik, M. (1981) Macrocyclic trichothecene toxins produced by a strain of Stachybotrys-Atra from Hungary. *Appl. Environ. Microbiol. 41*, 1428−1432.
(108) Kuisle, O., Lolo, M., Quiñoá, E., and Riguera, R. (1999) Monitoring the solid-phase synthesis of depsides and depsipeptides. A color test for hydroxyl groups linked to a resin. *Tetrahedron 55*, 14807−14812.

(109) Mori, H., Sato, T., Nagase, H., Sakai, Y., Yamaguchi, S., Iwata, Y., Hashimoto, R., Yamazaki, F., and Hayata, M. (1994) Rapid screening method for pesticides as the cause substances of toxicosis by TLC. *Jpn. J. Toxicol. Environ. Health 40*, 101−110.
(110) Sladek, N. E. (1973) Evidence for an aldehyde possessing alkylating activity as primary metabolite of cyclophosphamide. *Cancer Res. 33*, 651−658.
(111) Struck, R. F., Alberts, D. S., Horne, K., Phillips, J. G., Peng, Y.-M., and Roe, D. J. (1987) Plasma pharmacokinetics of cyclophosphamide and its cytotoxic metabolites after intravenous versus oral administration in a randomized, crossover trial. *Cancer Res. 47*, 2723−2726.
(112) Takitani, S., Asabe, Y., Kato, T., Suzuki, M., and Ueno, Y. (1979) Spectrodensitometric determination of trichothecene mycotoxins with 4-(*p*-nitrobenzyl)pyridine on silica-gel thin-layer chromatograms. *J. Chromatogr. 172*, 335−342.
(113) Tsunoda, N. (1986) Simultaneous determination of organophosphorus pesticides by thin-layer chromatography. *Jpn. J. Toxicol. Environ. Health 32*, 447−454.
(114) Trams, E. G. (1958) Determination of bis-(beta-chloroethyl) amines and related compounds with 8-quinolinol. *Anal. Chem. 30*, 256−259.
(115) Mason, W. A., and Meloan, C. E. (1976) Degradation products of phoxim (Bay-77488) on stored wheat. *J. Agric. Food Chem. 24*, 299−304.
(116) Tee, K. L., and Schwaneberg, U. (2006) A screening system for the directed evolution of epoxygenases: Importance of position 184 in P450 BM3 for stereoselective styrene epoxidation. *Angew. Chem., Int. Ed. 45*, 5380−5383.
(117) Doderer, K., Lutz-Wahl, S., Hauer, B., and Schmid, R. D. (2003) Spectrophotometric assay for epoxide hydrolase activity toward any epoxide. *Anal. Biochem. 321*, 131−134.
(118) Zocher, F., Enzelberger, M. M., Bornscheuer, U. T., Hauer, B., and Schmid, R. D. (1999) A colorimetric assay suitable for screening epoxide hydrolase activity. *Anal. Chim. Acta 391*, 345−351.
(119) Eder, E., and Kutt, W. (1989) The dependence of the mutagenicity of methanesulfonic acid-esters in S-typhimurium TA100 on the alkylation mechanism. *Chem.-Biol. Interact 69*, 45−59.
(120) Eder, E., Espinosa-González, J., Mayer, A., Reichenberger, K., and Boerth, D. (2006) Autoxidative activation of the nematocide 1,3-dichloropropene to highly genotoxic and mutagenic derivatives: Consideration of genotoxic/carcinogenic mechanisms. *Chem. Res. Toxicol. 19*, 952−959.
(121) Eder, E., Kutt, W., and Deininger, C. (2006) In vitro induction of micronuclei by monofunctional methanesulphonic acid esters: Possible role of alkylation mechanisms. *Chem.-Biol. Interact. 164*, 76−84.
(122) Van Duuren, B. L. (1969) Carcinogenic epoxides, lactones, and halo-ethers and their mode of action. *Ann. N.Y. Acad. Sci. 163*, 633−650.
(123) Lawley, P. D., Orr, D. J., and Jarman, M. (1975) Isolation and identification of products from alkylation of nucleic-acids-ethyl-purines and isopropyl-purines. *Biochem. J. 145*, 73−84.
(124) Ho, T. L. (1975) Hard soft acids bases (HSAB) principle in organic chemistry. *Chem. Rev. 75*, 1−20.
(125) Offermanns, S. (2008) *Encyclopedia of Molecular Pharmacology*, Springer, New York.
(126) Pratihar, S., and Roy, S. (2010) Nucleophilicity and site selectivity of commonly used arenes and heteroarenes. *J. Org. Chem. 75*, 4957−4963.
(127) Exner, O. (1988) *Correlation Analysis of Chemical Data*, Plenum, New York.
(128) Swain, C. G., and Scott, C. B. (1953) Quantitative correlation of relative rates. Comparison of hydroxide ion with other nucleophilic reagents toward alkyl halides, esters, epoxides and acyl halides. *J. Am. Chem. Soc. 75*, 141−147.
(129) Ritchie, C. D. (1972) Nucleophilic reactivities toward cations. *Acc. Chem. Res. 5*, 348−354.
(130) Ritchie, C. D. (1975) Cation-anion combination reactions. XIII. Correlation of the reactions of nucleophiles with esters. *J. Am. Chem. Soc. 97*, 1170−1179.
(131) Mayr, H., and Patz, M. (1994) Scales of nucleophilicity and electrophilicity: a system for ordering polar organic and organometallic reactions. *Angew. Chem., Int. Ed. 33*, 938−957.
(132) Lawley, P. D., and Thatcher, C. J. (1970) Methylation of deoxyribonucleic acid in cultured mammalian cells by N-methyl-N′-nitro-N-nitrosoguanidine - influence of cellular thiol concentrations on extent of methylation and 6-oxygen atom of guanine as a site of methylation. *Biochem. J. 116*, 693−&.
(133) Swenson, D. H., and Lawley, P. D. (1978) Alkylation of deoxyribonucleic-acid by carcinogens dimethyl sulfate, ethyl methanesulfonate, N-ethyl-N-nitrosourea and N-methyl-N-nitrosourea - relative reactivity of phosphodiester site thymidylyl(3′-5′)thymidine. *Biochem. J. 171*, 575−587.
(134) Singer, B., and Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York.
(135) Loechler, E. L. (1994) A violation of the Swain-Scott principle, and not SN1 versus SN2 reaction mechanisms, explains why carcinogenic alkylating agents can form different proportions of adducts at oxygen versus nitrogen in DNA. *Chem. Res. Toxicol. 7*, 277−280.
(136) Beranek, D. T. (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat. Res.-Fundam. Mol. Mech. Mutag. 231*, 11−30.
(137) Meier, I., Shephard, S. E., and Lutz, W. K. (1990) Nitrosation of aspartic-acid, aspartame, and glycine ethylester: alkylation of 4-(*p*-nitrobenzyl)pyridine (NBP) in vitro and binding to DNA in the rat. *Mutat. Res. 238*, 193−201.
(138) Boerth, D. W., Eder, E., Rasul, G., and Morais, J. (1991) Theoretical structure-activity study of mutagenic allyl chlorides. *Chem. Res. Toxicol. 4*, 368−372.
(139) Nakano, N., Yamamoto, A., Kobayashi, Y., and Nagashima, K. (1995) Development of a monitoring tape for phosgene in air. *Talanta 42*, 641−645.
(140) Barawkar, D. A., and Ganesh, K. N. (1995) Fluorescent d(CGCGAATTCGCG): characterization of major groove polarity and study of minor groove interactions through a major groove semantophore conjugate. *Nucleic Acids Res. 23*, 159−164.
(141) Dierickx, K. M. E., Journé, F., Gerbaux, P., Morandini, R., Kauffmann, J.-M., and Ghanem, G. E. (2009) Improving the spectrophotometric determination of the alkylating activity of anticancer agents: A new insight into the mechanism of the NBP method. *Talanta 77*, 1370−1375.
(142) Groenen, P. J., and Busink, E. (1988) Alkylating activity in food products-especially sauerkraut and sour fermented dairy products-after incubation with nitrite under quasi-gastric conditions. *Food Chem. Toxicol. 26*, 215−225.
(143) Agarwal, S. C., and Neton, J. (1989) Mutagenicity and alkylating activity of the aqueous chlorination products of humic-acid and their molecular-weight fractions. *Sci. Total Environ. 79*, 69−83.
(144) Katagi, M., Tsuchihashi, H., Hanada, S., Jinmori, H., and Otsuki, K. (1993) Determination of dichlorvos, trichlorfon and their metabolites and degradation products in environmental water, drinks and human urine. *Jpn. J. Toxicol. Environ. Health 39*, 459−468.
(145) Berck, B., Iwata, Y., and Gunther, F. A. (1981) Worker environment research: Rapid field method for estimation of organophosphorus insecticide residues on citrus foliage and in grove soil. *J. Agric. Food Chem. 29*, 209−216.
(146) Tingfa, D., Shiguang, Z., and Mousheng, T. (1990) Estimation method of residual alkylating agents in water treated with chloring-containing oxidant. *Water, Air, Soil Pollut. 49*, 63−67.
(147) Guth, J. A., and Manner, L. P. (1967) 4-(P-Nitrobenzyl)-Pyridin als Reagens zur Dunnschichtchromatographischen Bestimmung verschiedener Azaheterozyklen. *J. Chromatogr. 30*, 514−521.
(148) Asami, M., Nakamura, K.-I., Kawada, K., and Tanaka, M. (1979) Evaluation of the alkylating activity of nitrosoureas by thin-layer densitometry. *J. Chromatogr. 174*, 216−220.

(149) Guengerich, F. P., Geiger, L. E., Hogy, L. L., and Wright, P. L. (1981) In vitro metabolism of acrylonitrile to 2-cyanoethylene oxide, reaction with glutathione, and irreversible binding to proteins and nucleic acids. *Cancer Res. 41*, 4925−4933.
(150) Fox, B. G., Borneman, J. G., Wackett, L. P., and Lipscomb, J. D. (1990) Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus-trichosporium* Ob3b - mechanistic and environmental implications. *Biochemistry 29*, 6419−6427.
(151) Newman, L. M., and Wackett, L. P. (1997) Trichloroethylene oxidation by purified toluene 2-monooxygenase: Products, kinetics, and turnover-dependent inactivation. *J. Bacteriol. 179*, 90−96.
(152) Forkert, P. G., Premdas, P. D., and Bowers, R. J. (2000) Epoxide formation from diallyl sulfone is associated with CYP2E1 inactivation in murine and human lungs. *Am. J. Respir. Cell Mol. Biol. 23*, 687−695.
(153) Premdas, P. D., Bowers, R. J., and Forkert, P. G. (2000) Inactivation of hepatic CYP2E1 by an epoxide of diallyl sulfone. *J. Pharmacol. Exp. Ther. 293*, 1112−1120.
(154) Tan, Y. L., and Cole, D. R. (1965) New method for determination of alkylating agents in biologic fluids. *Clin. Chem. 11*, 58−62.
(155) Cox, P. J., and Farmer, P. B. (1975) Effect of plasma on reaction of cyclophosphamide with 4-(*p*-nitrobenzyl)pyridine. *Chem.-Biol. Interact. 10*, 103−114.
(156) Christian, R. A., Chaffee, S. K., Hovick, C. J., and Steele, W. J. (1980) A stable colorimetric assay for cyclophosphamide and its alkylating metabolites based on the alkylation of 4-(4′-nitrobenzyl)-pyridine. *Life Sci. 27*, 2595−2599.
(157) Jiang, H. B., Ichikawa, M., Furukawa, A., Tomita, S., and Ichikawa, Y. (2000) Reductive activation of mitomycin C by neuronal nitric oxide synthase. *Biochem. Pharmacol. 60*, 571−579.
(158) Mellett, L. B., and Woods, L. A. (1960) The comparative physiological disposition of ThioTEPA and TEPA in the dog. *Cancer Res. 20*, 524−532.
(159) Huijbrechts, A. M. L., Haar, R. t., Schols, H. A., Franssen, M. C. R., Boeriu, C. G., and Sudhölter, E. J. R. (2011) Synthesis and application of epoxy starch derivatives. *Carbohydr. Polym. 79*, 858−866.
(160) Magdalou, J., and Hammock, B. D. (1988) 1,2-Epoxycycloalkanes: Substrates and inhibitors of microsomal and cytosolic epoxide hydrolases in mouse liver. *Biochem. Pharmacol. 37*, 2717−2722.
(161) Rink, R., Fennema, M., Smids, M., Dehmel, U., and Janssen, D. B. (1997) Primary structure and catalytic mechanism of the epoxide hydrolase from Agrobacterium radiobacter AD1. *J. Biol. Chem. 272*, 14650−14657.
(162) Bhatnagar, T., Manoj, K. M., and Baratti, J. C. (2001) A spectrophotometric method to assay epoxide hydrolase activity. *J. Biochem. Biophys. Methods 50*, 1−13.
(163) Alcalde, M., Farinas, E. T., and Arnold, F. H. (2004) Colorimetric high-throughput assay for alkene epoxidation catalyzed by cytochrome P450 BM-3 variant 139-3. *J. Biomol. Screen. 9*, 141−146.
(164) Cedrone, F., Bhatnagar, T., and Baratti, J. C. (2005) Colorimetric assays for quantitative analysis and screening of epoxide hydrolase activity. *Biotechnol. Lett. 27*, 1921−1927.
(165) Zocher, F., Enzelberger, M. M., Bornscheuer, U. T., Hauer, B., Wohlleben, W., and Schmid, R. D. (2000) Epoxide hydrolase activity of Streptomyces strains. *J. Biotechnol. 77*, 287−292.
(166) Kim, H. S., Lee, O. K., Lee, S. J., Hwang, S., Kim, S. J., Yang, S. H., Park, S., and Lee, E. Y. (2006) Enantioselective epoxide hydrolase activity of a newly isolated microorganism, *Sphingomonas echinoides* EH-983, from seawater. *J. Mol. Catal. B 41*, 130−135.
(167) Li, N., Zhang, Y., and Feng, H. (2009) Biochemical characterization and transcriptional analysis of the epoxide hydrolase from white-rot fungus phanerochaete chrysosporium. *Acta Biochim. Biophys. Sin. 41*, 638−647.
(168) Zhang, L. F., Wu, J. M., and Feng, H. (2011) Homology modelling and site-directed mutagenesis studies of the epoxide hydrolase from *Phanerochaete chrysosporium*. *J. Biochem. 149*, 673−684.
(169) Kumar, R., Wani, S. I., Chauhan, N. S., Sharma, R., and Sareen, D. (2011) Cloning and characterization of an epoxide hydrolase from *Cupriavidus metallidurans*-CH34. *Protein Expression Purif. 79*, 49−59.
(170) Kubo, T., Peters, M. W., Meinhold, P., and Arnold, F. H. (2006) Enantioselective epoxidation of terminal alkenes to (R)- and (S)-epoxides by engineered cytochromes P450 BM-3. *Chemistry 12*, 1216−1220.
(171) Qaed, A. A., Lin, H., Tang, D. F., and Wu, Z. L. (2011) Rational design of styrene monooxygenase mutants with altered substrate preference. *Biotechnol. Lett. 33*, 611−616.
(172) Eder, E., Henschler, D., and Neudecker, T. (1982) Mutagenic properties of allylic and *α,β*-unsaturated compounds: consideration of alkylating mechanisms. *Xenobiotica 12*, 831−848.
(173) Eya, B. K., and Talcott, R. E. (1980) Effect of N-Alkyl chain length on the mutagenicity of N-nitrosated 1-naphthyl N-alkylcarbamates. *Environ. Mol. Mutagen. 2*, 395−404.
(174) Yano, K., and Isobe, M. (1979) Mutagenicity of N-methyl-N′-aryl-N-nitrosoureas and N-methyl-N′-aryl-N′-methyl-N-nitrosoureas in relation to their alkylating activity. *Cancer Res. 39*, 5147−5149.
(175) Yano, K., Katayama, H., and Takemoto, K. (1984) Interrelationship between tumorigenicity and the chemical nature of N-methyl-N′-aryl-N-nitrosoureas. *Cancer Res. 44*, 1027−1030.
(176) Rosman, L. B., Chakraborty, P. K., Messerly, E. A., and Sinsheimer, J. E. (1988) Mutagenicity of aromatic glycidyl ethers with *Salmonella*. *Mutat. Res.-Genet. Toxicol. Environ. Mutag. 206*, 115−125.
(177) Rosman, L. B., Gaddamidi, V., and Sinsheimer, J. E. (1987) Mutagenicity of aryl propylene and butylene oxides with *Salmonella*. *Mutat. Res.-Genet. Toxicol. Environ. Mutag. 189*, 189−204.
(178) Isobe, M., and Yano, K. (1982) Comparison of mutagenicity and chemical-properties of N-methyl-N′-alkyl-N-nitrosoureas. *Mutat. Res. 93*, 57−66.
(179) Giri, A. K., Messerly, E. A., Chakraborty, P. K., Hooberman, B. H., and Sinsheimer, J. E. (1990) DNA strand breaks in liver for four aliphatic epoxides in mice. *Mutat. Res. 242*, 187−194.
(180) Schiffmann, D., Eder, E., Neudecker, T., and Henschler, D. (1983) Induction of unscheduled DNA synthesis in HeLa cells by allylic compounds. *Cancer Lett. 20*, 263−269.
(181) Wheeler, G. P., Bowdon, B. J., Grimsley, J. A., and Lloyd, H. H. (1974) Interrelationships of some chemical, physicochemical, and biological activities of several 1-(2-haloethyl)-1-nitrosoureas. *Cancer Res. 34*, 194−200.
(182) Ashby, J., Hilton, J., Dearman, R. J., Callander, R. D., and Kimber, I. (1993) Mechanistic relationship among mutagenicity, skin sensitization, and skin carcinogenicity. *Environ. Health Perspect. 101*, 62−67.
(183) Shyam, K., Penketh, P. G., Divo, A. A., Loomis, R. H., Rose, W. C., and Sartorelli, A. C. (1993) Synthesis and evaluation of 1-acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines as antineoplastic agents. *J. Med. Chem. 36*, 3496−3502.
(184) Jesson, M. I., Johnston, J. B., Robotham, E., and Begleiter, A. (1989) Characterization of the DNA-DNA cross-linking activity of 3′-(3-cyano-4-morpholinyl)-3′-deaminoadriamycin. *Cancer Res. 49*, 7031−7036.
(185) Freeman, J. A., Johnson, J. V., Yost, R. A., and Kuehl, D. W. (1994) Gas-phase ion−molecule reactions: a model for the determination of biologically reactive electrophilic contaminants in the environment. *Anal. Chem. 66*, 1902−1910.
(186) Beyer, U., Roth, T., Schumacher, P., Maier, G., Unold, A., Frahm, A. W., Fiebig, H. H., Unger, C., and Kratz, F. (1998) Synthesis and in vitro efficacy of transferrin conjugates of the anticancer drug chlorambucil. *J. Med. Chem. 41*, 2701−2708.
(187) Kratz, F., Beyer, U., Roth, T., Schütte, M. T., Unold, A., Fiebig, H. H., and Unger, C. (1998) Albumin conjugates of the anticancer drug chlorambucil: synthesis, characterization, and in vitro efficacy. *Arch. Pharm. 331*, 47−53.
(188) Dipple, A., and Slade, T. A. (1970) Structure and activity in chemical carcinogenesis: Reactivity and carcinogenicity of 7-bromomethylbenz[a]anthracene and 7-bromomethyl-12-methylbenz-[a]anthracene. *Eur. J. Cancer 6*, 417−423.

(189) Van Duuren, B. L., and Goldschmidt, B. M. (1966) Carcinogenicity of epoxides, lactones, and peroxy compounds. III. biological activity and chemical reactivity. *J. Med. Chem. 9*, 77−79.
(190) Walles, S. A. S. (1980) Determination of reaction-rate constants for alkylation of 4-(*p*-nitrobenzyl)pyridine by different alkylating-agents. *Toxicol. Lett. 5*, 161−167.
(191) Sims, P. (1972) Epoxy derivatives of aromatic polycyclic hydrocarbons: synthesis of dibenz[a,c]anthracene 10,11-oxide and its metabolism by rat-liver preparations. *Biochem. J. 130*, 27−35.
(192) Peterson, A. R., Peterson, H., Spears, C. P., Trosko, J. E., and Sevanian, A. (1988) Mutagenic characterization of cholesterol epoxides in Chinese hamster V79 cells. *Mutat. Res., Environ. Mutag. Relat. Subj. 203*, 355−366.
(193) Phillips, D. H., Grover, P. L., and Sims, P. (1978) Some properties of vicinal diol-epoxides derived from benz(a)anthracene and benzo(a)pyrene. *Chem.-Biol. Interact. 20*, 63−75.
(194) Lalka, D., and Bardos, T. J. (1975) Cyclophosphamide, 2,2-dimethylaziridines and other alkylating-agents as inhibitors of serum-cholinesterase. *Biochem. Pharmacol. 24*, 455−462.
(195) Anderson, W. K., and Heider, A. R. (1986) Vinylogous carbinolamine tumor inhibitors. 20. comparison of the chemical reactivities and antineoplastic activities of alpha,beta, alpha,beta′, beta,beta′, and alpha,alpha′-bis[[[(2-propylamino)carbonyl]oxy]-methyl]-substituted pyrroles. *J. Med. Chem. 29*, 2392−2395.
(196) Lin, E. L. C., Daniel, F. B., Herrenfreund, S. L., and Pereira, M. A. (1986) Haloacetonitriles: metabolism, genotoxicity, and tumor-initiating activity. *Environ. Health Perspect. 69*, 67−71.
(197) Manso, J. A., Pérez-Prior, M. T., Gómez-Bombarelli, R., González-Pérez, M., Céspedes, I. F., García-Santos, M. P., Calle, E., and Casado, J. (2008) Alkylating potential of *N*-phenyl-*N*-nitrosourea. *J. Phys. Org. Chem. 22*, 386−389.
(198) Peterson, L. A., Harris, T. M., and Guengerich, F. P. (1988) Evidence for an episulfonium ion intermediate in the formation of S-[2-(N7-guanyl)ethyl]glutathione in DNA. *J. Am. Chem. Soc. 110*, 3284−3291.
(199) García-Santos, M. P., González-Mancebo, S., Hernández-Benito, J., Calle, E., and Casado, J. (2002) Reactivity of amino acids in nitrosation reactions and its relation to the alkylating potential of their products. *J. Am. Chem. Soc. 124*, 2177−2182.
(200) García-Santos, M. P., Calle, E., and Casado, J. (2001) Amino acid nitrosation products as alkylating agents. *J. Am. Chem. Soc. 123*, 7506−7510.
(201) Casado, J., López-Quintela, M. A., and Lorenzo-Barral, F. M. (1986) The initial rate method in chemical kinetics: Evaluation and experimental illustration. *J. Chem. Educ. 63*, 450.
(202) Karchesy, J. J., Arbogast, B., and Deinzer, M. L. (1987) Kinetics of alkylation reactions of pyrrolizidine alkaloid pyrroles. *J. Org. Chem. 52*, 3867−3872.
(203) Cooper, R. A., and Huxtable, R. J. (1999) The Relationship between Reactivity of Metabolites of Pyrrolizidine Alkaloids and Extrahepatic Toxicity, in *Proceedings of the Forty-Second Annual Meeting of the Western Pharmacology Society* (Huxtable, R., Ed.) WPS, Reno, NV, pp 13−16.
(204) Barbin, A., Bereziat, J. C., Croisy, A., Oneill, I. K., and Bartsch, H. (1990) Nucleophilic selectivity and reaction-kinetics of chloroethylene oxide assessed by the 4-(*p*-nitrobenzyl)pyridine assay and proton nuclear magnetic resonance spectroscopy. *Chem.-Biol. Interact. 73*, 261−277.
(205) Giraud, I., Rapp, M., Maurizis, J.-C., and Madelmont, J.-C. (2002) Synthesis and in vitro evaluation of quaternary ammonium derivatives of chlorambucil and melphalan, anticancer drugs designed for the chemotherapy of chondrosarcoma. *J. Med. Chem. 45*, 2116−2119.
(206) Kagn, S. I., and Spears, C. P. (1987) Linear free energy relationships and cytotoxicities of para-substituted 2-haloethyl aryl selenides and bis(2-chloroethyl) selenides. *J. Med. Chem. 30*, 597−602.
(207) Gómez-Bombarelli, R., González-Pérez, M., Pérez-Prior, M. T., Manso, J. A., Calle, E., and Casado, J. (2008) Chemical reactivity and biological activity of diketene. *Chem. Res. Toxicol. 21*, 1964−1969.
(208) Pérez-Prior, M. T., Manso, J. A., Gómez-Bombarelli, R., González-Perez, M., García-Santos, M. P., Calle, E., Caballero, M. C., and Casado, J. (2008) Reactivity of some products formed by the reaction of sorbic acid with sodium nitrite: decomposition of 1,4-dinitro-2-methylpyrrole and ethylnitrolic acid. *J. Agric. Food Chem. 56*, 11824−11829.
(209) Gómez-Bombarelli, R., González-Pérez, M., Pérez-Prior, M. T., Manso, J. A., Calle, E., and Casado, J. (2009) Kinetic study of the neutral and base hydrolysis of diketene. *J. Phys. Org. Chem. 22*, 438−442.
(210) Crick, F. H. C., Wang, J. C., and Bauer, W. R. (1979) Is DNA really a double helix? *J. Mol. Biol. 129*, 449−457.
(211) Vogel, E. W., and Nivard, M. J. M. (1994) The subtlety of alkylating agents in reactions with biological macromolecules. *Mutat. Res.-Fundam. Mol. Mech. Mutag 305*, 13−32.
(212) Spears, C. P. (1981) Nucleophilic selectivity ratios of model and clinical alkylating agents by 4-(4′-nitrobenzyl)pyridine competition. *Mol. Pharmacol. 19*, 496−504.
(213) Schrader, T. J., Langlois, I., Soper, K., and Cherry, W. (2002) Mutagenicity of bisphenol A (4,4′-isopropylidenediphenol) in vitro: Effects of nitrosylation. *Teratog. Carcinog. Mutagen. 22*, 425−441.
(214) Thomas, J. J., Kim, J. H., and Mauro, D. M. (1992) 4-(4-Nitrobenzyl)pyridine tests for alkylating-agents following chemical oxidative activation. *Arch. Environ. Contam. Toxicol. 22*, 219−227.
(215) Sasaki, Y. F., Saga, A., Akasaka, M., Ishibashi, S., Yoshida, K., Su, Y. Q., Matsusaka, N., and Tsuda, S. (1998) Detection of in vivo genotoxicity of haloalkanes and haloalkenes carcinogenic to rodents by the alkaline single cell gel electrophoresis (comet) assay in multiple mouse organs. *Mutat. Res., Genet. Toxicol. Environ. Mutagen. 419*, 13−20.
(216) Brendler-Schwaab, S., Hartmann, A., Pfuhler, S., and Speit, G. (2005) The in vivo comet assay: use and status in genotoxicity testing. *Mutagenesis 20*, 245−254.
(217) Recio, L., Hobbs, C., Caspary, W., and Witt, K. L. (2010) Dose-response assessment of four genotoxic chemicals in a combined mouse and rat micronucleus (MN) and Comet assay protocol. *J. Toxicol. Sci. 35*, 149−162.
(218) Pu, X., Kamendulis, L. M., and Klaunig, J. E. (2009) Acrylonitrile-induced oxidative stress and oxidative DNA damage in male Sprague-Dawley rats. *Toxicol. Sci. 111*, 64−71.
(219) Shi, J., Krsmanovic, L., Bruce, S., Kelly, T., Paranjpe, M., Szabo, K., Arévalo, M., Atta-Safoh, S., Debelie, F., LaForce, M. K., Sly, J., and Springer, S. (2011) Assessment of genotoxicity induced by 7,12-dimethylbenz(a)anthracene or diethylnitrosamine in the Pig-a, micronucleus and Comet assays integrated into 28-day repeat dose studies. *Environ. Mol. Mutag. 52*, 711−720.
(220) Sasaki, Y. F., Saga, A., Akasaka, M., Ishibashi, S., Yoshida, K., Su, Y. Q., Matsusaka, N., and Tsuda, S. (1998) Organ-specific genotoxicity of the potent rodent colon carcinogen 1,2-dimethylhydrazine and three hydrazine derivatives: difference between intraperitoneal and oral administration. *Mutat. Res., Genet. Toxicol. Environ. Mutagen. 415*, 1−12.

NOTE ADDED AFTER ISSUE PUBLICATION

An error occurred in the PDF version of this review when issue 6 was published on June 18, 2012, which resulted in extra space on page 1186 and the disappearance of references 189−220 on page 1191. The extra space on page 1186 was removed, and references 189−220 were added back into page 1191. The corrected version was published on July 20, 2012.