The Use of Biochemical and Molecular Parameters to Estimate Dose–Response Relationships at Low Levels of Exposure

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Biomarkers based on alterations in molecular and biochemical parameters may be useful in chemical risk assessment for establishing the presence of an exposure, ranking relative risks among exposed individuals, and estimating risks at low levels of exposure. Because it is unlikely that the relation between toxic responses and the degree of alteration in the biomarker is equivalent at all doses, quantification of risks at low levels is not necessarily more accurate using these biomarkers for extrapolation. The application of response biomarkers for risk evaluation at low levels of exposure is discussed in relation to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a compound that causes induction of cytochromes CYP1A1 and CYP1A2 in liver and other tissues. CYP1A1 induction in liver increases monotonically with TCDD dosage; however, several of the dose–response curves for hepatic effects of TCDD are U-shaped. The U-shaped dose–response curve for hepatic tumor promotion appears to result because the integrated toxicologic response depends on multiple underlying processes—mitosis suppression, toxicity, and cell proliferation—each of which has a different dose–response relationship with respect to TCDD. Although dose–response relationships for the biomarkers are not expected to duplicate the complex shapes seen with the integrated responses, measurements and pharmacodynamic modeling of the changes in these molecular and biochemical parameters can still be useful for obtaining an upper-bound risk estimate at low levels of exposure. — Environ Health Perspect 106(Suppl 1):349-355 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-1/349-355andersen/abstract.html

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Introduction

Epidemiologic studies in human populations are expected to be more relevant for human risk assessments than the results of animal toxicity studies. However, because of confounding factors related to concurrent exposures to other chemicals, differences in individual lifestyles, difficulties in accurately reconstructing exposures, and the small sizes of most cohorts, few epidemiologic studies provide results that unambiguously determine risks to humans at low levels of exposure. Thus, most contemporary risk assessments are based on results from animal studies, in which it is possible to control the exposure conditions and expose animals to high doses of overtly toxic compounds.

In these cases, accurate estimation of risks to human health posed by low-level exposure to potentially toxic compounds requires knowledge of the dose–response relationship over a broad range of exposures in the animal species. Unfortunately, dose–response curves that define risks in animals at low response rates are almost nonexistent and in some cases may be impossible to obtain. For toxic responses with measurable background rates, such as cancer and teratogenesis, it is difficult to assess the significance of small increases above background response rates. These studies only infrequently resolve differences of response in the 1 in 20 to 1 in 100 range. When evaluating impaired function such as reproductive competence, the natural variability in a healthy population of measures of effect such as numbers of live births per litter greatly restrict accurate assessment of low-level response rates related to chemical exposures.

It may be possible to extend dose–response curves to lower levels of exposure based on theoretical knowledge of the mechanisms of toxicity by using so-called biologically based models or by measuring precursor biochemical or molecular events directly involved in the sequence of events leading to toxicity states. Proposed revised carcinogen risk assessment guidelines (1) discuss the use of these precursor events to extend the dose–response curve to low levels of exposure and the role of biologically based models in cancer risk assessment. Precursor events should be causally related to toxicity to be useful in risk assessment. A precursor response linked mechanistically to toxicity is equivalent to a biomarker of response.

Biomarkers in Risk Assessment

Biomarkers are used to indicate that an individual has been exposed to a toxic chemical (exposure biomarkers) or that an individual is at some risk of toxicity because of the exposure (response biomarkers). Biomarkers of exposure include the presence of the compound or a specific metabolite in blood or tissues or the presence of macromolecular reaction products such as hemoglobin adducts, which are not related causally to any adverse outcome. Blood cholinesterase inhibition by organophosphate insecticides is considered a biomarker of exposure because the inhibition of this enzymatic activity is not believed to be involved directly in the neurotoxicity of these compounds. These exposure biomarkers can be important in epidemiologic studies for stratifying individuals according to intensity of exposure rather than relying on analysis of work practices or lifestyle factors in creating categories of exposure intensities.

With DNA-reactive carcinogenic compounds, response biomarkers include the presence of chemical-related DNA adducts and the identification of specific mutations in oncogenes. With compounds that serve as tumor promoters, exposure
may lead to altered regulation of growth regulatory genes or to recurrent toxicity with concomitant reparative hyperplasia. With either DNA-reactive or indirect-acting carcinogens, the various response biomarkers can be viewed in either qualitative or quantitative fashion.

If there is no evidence of an increase in response biomarkers, there should be no increase in expected risk from a toxic compound in target populations. This application provides a qualitative argument of the relationship between the biomarker and the presumed risk. However, based on the statistical power of the test measurement, there is still some level of increase in the biomarker and therefore some level of increased risk that cannot be evaluated based directly on the measurements. A more difficult question involves the quantitative relationship between the intensity of the biomarker in the organism and the risk of overt toxicity. To use these precursor events in quantitative risk estimation for low-level exposures, it is necessary to understand the relationship between these precursor changes and toxic responses over the full dose range of interest. This paper, using experience with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as an example, examines the advantages and disadvantages of using response biomarkers to estimate dose-response relationships quantitatively at low levels of exposures.

**Response Markers with TCDD**

TCDD is a member of the class of polychlorinated dibenzo-p-dioxins (PCDDs). Many of these PCDDs, especially those with lateral chlorine substituents, produce a characteristic constellation of biologic effects in experimental animals; these effects include liver cancer in female rats (2,3), teratogenicity (4) and immunotoxicity in mice (5), and reproductive/developmental effects in adult male and female rats exposed in utero (6,7). Cancer is a relatively high dose effect compared to these other responses.

**TCDD Biomarkers**

In addition to these overt toxic effects, TCDD also produces changes at the molecular and biochemical levels, including increases in the concentrations of a number of enzymes involved in cellular metabolism. The most extensively studied effect is the induction of two cytochrome P450 enzymes, CYP1A1 and CYP1A2, in multiple animal species. The toxicologic and enzyme-inducing effects of TCDD appear to be initiated by the interaction of TCDD with a cellular protein—the aryl hydrocarbon (Ah) receptor.

Our understanding of the mechanisms of TCDD interactions with the Ah receptor are based primarily on studies of CYP1A1 induction by TCDD. The inactive Ah receptor consists of an aggregate of several proteins (8).

Binding TCDD to the Ah receptor aggregate leads to dissociation of the Ah receptor from the aggregate and dimerization of the Ah–TCDD complex with another protein, the aryl hydrocarbon nuclear transferase (Arsnt). Both the Ah protein (9) and Arnt (10) are β-helix–loop–helix DNA binding proteins. The heterodimer formed from these two proteins has a high affinity for specific DNA response elements found upstream from the CYP1A1 gene. For other proteins, such as CYP1A2, gene transcription is increased by the Ah–TCDD complex even though there is no CYP1A1-like response element upstream from this gene (11).

**Other Hepatic Response Markers**

TCDD also has other effects in the liver intermediate between these biochemical responses and overt carcinogenicity. Pitot et al. (12) evaluated the ability of TCDD to promote growth of altered enzyme foci in livers of female rats initiated by a regimen of two-thirds partial hepatectomy (PH) followed by a dose of 10 mg diethylnitrosamine (DEN) per kg body weight. TCDD was a potent promoter in this study, causing an increase in the number of foci per volume and in the amount of the liver occupied by foci at daily doses of 100 ng TCDD/kg. The dose–response curve for these effects appeared to be U-shaped (Figure 1).

Van Bergelen et al. (13) found that TCDD treatment increased the concentrations of porphyrins in the liver and that this increase correlated well with the induction of CYP1A2. Mechanistically, this correlation may arise because the metabolism of uroporphyrinogen III to uroporphyrin III is catalyzed by CYP1A2 (14). TCDD also increases the proliferation rate of hepatocytes and induces toxicity, defined as cytoplasmic vacuolation, fatty changes, bile duct hyperplasia, and pigment in Kupfer cells (15). Stinchcombe et al. (16) found that apoptotic rates of the cells in altered foci staining positively for glutathione S-transferase (GST) produced by TCDD treatment were much lower than the apoptotic rates measured in these foci in the absence of TCDD. Any of these various processes may be regarded as precursor steps to the development of liver tumors.

**Specificity and Sensitivity**

Two important concepts in evaluating the utility of any particular biomarker are whether the biomarker can be associated uniquely with a specific exposure (specificity) and whether it is clearly elevated at even low levels of exposure (sensitivity). The presence of TCDD in tissues is a specific measurement of TCDD exposure because TCDD is not an endogenous compound in plants or animals. All the response markers—induction, cell proliferation, toxicity, and altered apoptosis—are nonspecific, i.e., they can be associated with other xenobiotics or with natural processes within the animal. Although all these response markers are nonspecific, some markers of induction of CYP1A enzymes, for example, are much more sensitive than others, cell proliferation and toxicity, for example.

The most widely used marker in experimental animals for exposure to PCDDs and similar compounds is the induction of the CYP1A family cytochromes. This sensitive biomarker is not specific to TCDD or even to halogenated polyaromatic compounds. Other PCDDs,
chlorinated dibenzofurans, some PCBs, and some natural products such as polynuclear aromatic hydrocarbons also induce these proteins. Within a human population, increased CYP1A1 is a measure of exposure to a wide variety of compounds that activate the Ah-receptor aggregate.

Characterizing the Biomarker Dose Response

Induction of CYP1A1 mRNA by TCDD has been studied to assess responses at low levels of exposure in rats (17). The utility of CYP1A1 as a response marker at low levels of exposure requires accurate characterization of the dose–response relationship between induction and hepatic concentrations of TCDD.

Bars and Elcombe (18) first noted that CYP1A enzyme induction by TCDD was heterogeneous over the liver acinus. Tritscher et al. (19) reported induction in livers of rats treated for 3 months. At lower doses (3.5 and 10.7 ng TCDD/kg/day) induction occurred in the centrilobular region and progressively moved outward to the mid-zonal and then the periporal areas as dose increased up to 125 ng/kg/day. In addition, when the immunohistochemically stained slides were evaluated closely, cells were found to be either fully induced or in a basal state, i.e., there was a sharp boundary between areas of induced cells and areas with no induced cells (18). Thus, TCDD appears to cause a shift in the state of hepatocytes from the basal noninduced phenotype to a dioxin-activated phenotype in which CYP1A1 and CYP1A2 are fully induced.

Physiologically based pharmacokinetic models for TCDD that included gene induction were developed in the late 1980s and early 1990s (20–24). These initial CYP1A family induction models did not consider heterogeneous induction in evaluating the dose response for this molecular marker of the effects of TCDD on the liver. These earlier models were successful in explaining the induction averaged over the whole liver but required alteration to simultaneously describe the nonlinear induction of CYP1A1 mRNA by TCDD (17).

Models of Regional Induction

These pharmacokinetic and gene induction models recently were extended to consider regional effects together with the observation of the sharp boundary between induced and noninduced regions (25,26). In common with their single liver compartment precursors, these more recent models still described TCDD activity based on the presumption of an increased rate of transcription of CYP1A mRNA related to the occupancy of dioxin response elements (DREs) on DNA by the Ah–TCDD complex. The relationship between Ah–TCDD and the rate of transcription was:

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rate = rate_{basal} + \frac{rate_{max} \times Ah \text{- TCDD}^n}{Ah \text{- TCDD}^n + K_{di}^n}
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To account for regional induction with sharp boundaries, the presumed binding affinity of DREs for the Ah–TCDD complex (Kdi) were varied among five compartments in the liver. The five compartments (Figure 2) were defined geometrically as a hexagonal acinar structure (25). The model was capable of simulating available data on total induction of mRNA and CYP1A family proteins and on the regional patterns of induction when the Hill coefficients were large (4 or greater) and the Kdi values varied by a factor of 3 between compartments. This parameterization of the multicompartment liver model predicted a variation of 81 in the effective binding affinity over the five compartments.

The requirement of a large n-value indicates a steep, highly nonlinear dose–response relationship for induction in both the observed and low-dose regions. In the published analysis by Andersen et al. (26) the dissociation constant in the centrilobular region (the most sensitive region of the liver for CYP1A induction) was estimated to be 0.11 nM Ah–TCDD for induction of CYP1A2. With the n-value equal to 4, the proportionate response for CYP induction in the centrilobular region at a 10-fold lower concentration of Ah–TCDD complex (0.011 nM) would be reduced to 1 × 10⁻⁴. The response rate for the centrilobular hepatocytes would fall to one dioxin-activated cell per 1,000,000 normal cells when the concentration of the Ah–TCDD complex in this compartment is the Ah–TCDD complex–DNA dissociation constant divided by 31.

Thus, risks associated with events that were directly dependent on induction and activation of cells to a dioxin-responding phenotype as obligate precursor event would be minimal (1 in 10⁻⁶) if the receptor complex concentration fell below 0.000355 nM. Similar conclusions regarding receptor–ligand concentrations associated with minimal risks would also be valid for other hepatic enzyme inducers with heterogeneous patterns of induction in which individual cells appear to be either in the normal state or fully induced. These compounds include phenobarbital, peroxisomal proliferators, and a variety of dioxinlike compounds, including dibenzofurans and coplanar PCBs.

Figure 2. A schematic representation of the surfaces of hexagonal acinar structures within the liver in the geometric liver model used to predict regional induction characteristics with TCDD. The most recent physiologically based pharmacokinetic (PBPK) models for TCDD divide the liver acinus into five compartments based on concentric regions within the acinus (25,26). This geometric model (A) allows prediction of regional induction (B) by assuming different binding characteristics of TCDD with DNA response elements in each region. Estimates of total induction in a region are estimated and compared to maximally induced levels. The proportion induction is then used to calculate a color intensity for the region (B). To obtain sharp boundaries with some fully induced regions (the two centrilobular zones) and other noninduced regions (the two periporal zones), the Hill coefficients in the induction equations in the PBPK models must be 4 or greater. Any comprehensive model of hepatic induction by most CYP450 inducers should account for regional effects as well as effects averaged over the entire liver.
Biomarkers and Low-dose Risks

Enzyme induction indicates that hepatocytes have responded to TCDD exposure and provides a measure of the number of hepatocytes in the new dioxin-activated state. Induction alone does not appear to be a toxic response or to be directly related to toxicity, although the induction of p53-phya associated with increased CYP1A2 may be an exception.

The hepatocarcinogenicity of dioxin appears instead to result from the spontaneous appearance of mutated cells that have reduced responsiveness to the growth regulatory control affecting normal cells. In the presence of TCDD, mutated cells grow to be identifiable clones. As the clones increase in size, the opportunities for subsequent mutations to more aggressive cell types also increase. If the probability of mutation or the cell proliferation rates were simple functions of the cells recruited into the dioxin-activated state, it would be possible to estimate the dose-response curve for all levels of exposure from measurements of CYP1A1 induction. The evidence below, however, suggests that no simple relationship exists between carcinogenicity and the extent of enzyme induction.

Cell-labeling Index/Regional Proliferation

Fox et al. (27) found that rats treated for 2 weeks with TCDD at doses leading to tissue concentrations of 150 ng/g tissue exhibited marked shifts in patterns of hepatocyte proliferation within the acinar structures of the liver. In control rats proliferation occurred randomly throughout the acinus. With TCDD treatment, the proliferation was primarily periportal, with reduced proliferation rates in the rest of the liver. Maronpot et al. (15) found that in TCDD-treated rats proliferating cells were preferentially located in the periporal area of the acinus at doses of 10, 35, and 125 ng/kg/day. The authors also noted a decrease in labeling index in DEN-initiated livers treated with 3.5 ng TCDD/kg/day, which suggested inhibition of cell proliferation compared to that in controls at this relatively low dose of TCDD (Figure 3).

Bauman et al. (28) studied the effect of TCDD on the regeneration of liver after PH. Twenty-four hours after PH, 61% of the hepatocytes in control animals were in the cell cycle, i.e., cells identified by staining techniques to be in either G1, S, G2, or M phase of the cell cycle. With TCDD only 41% of the hepatocytes were in the cell cycle at a comparable time after PH. These results indicate that one of the functional responses of the liver to TCDD is a decrease in responsiveness of hepatocytes to mitogenic stimulation. The authors also found a decreased response of TCDD-treated livers to mitostimulation by lead nitrate. In general, the body of research with TCDD indicated mitosuppression and shift of proliferation to the periporal region at low doses, whereas cell proliferation throughout the liver and toxicity occur at higher doses.

Mechanisms of Promotion and Carcinogenicity

Two proposals have been made to account for the potency of TCDD as a tumor promoter. Portier et al. (29) and Moolgavkar et al. (30) developed models of promotion in which DEN treatment produced a single type of mutated cell. All clones observed at each dose of TCDD were believed to be derived from that single cell type. Based on this biologic structure for the nature of the mutated cell population, the initiation-promotion data were consistent with a model in which TCDD acted as an initiator to increase the production of mutated cells over time during the study. This conclusion is difficult to reconcile with the fact that TCDD has been tested for mutagenicity in multiple in vivo and in vitro test systems. With two exceptions (31,32) all mutagenicity tests were negative.

In contrast, Andersen et al. (33) and Conolly and Anderson (34) evaluated an alternative model for interpreting these initiation-promotion results with TCDD. In their model DEN initiation produces two cell types (A and B) capable of becoming clones of enzyme-altered foci in the liver. One of these cell types, A, responds to the negative growth environment associated with TCDD treatment. The net growth rate for this cell type, given by the birth rate minus the death rate (α\(b\) - \(βb\)), decreases with increasing TCDD exposure. Most clones observed in DEN controls in the absence of TCDD treatment are expected to be derived from these A cells. The second type of cell, B, is unresponsive to the mitoinhibitory environment associated with TCDD treatment. For these cells \(α_B - β_B\) increases with increasing concentrations of TCDD. At the high exposure concentrations in the initiation-promotion studies, the observed clones are derived primarily from these B cells. This two-cell model is consistent with the observed U-shaped dose-response curves, explains TCDD promotion without assuming a mutational component to the formation of the clones over time, and is consistent with a mitoinhibitory action of TCDD on normal cells that is absent in the B-cell clones (34).

Dose Dependencies of Response Mechanisms

The data on hepatic effects of TCDD are consistent with three distinct ranges of behavior (Table 1). Lower doses (1–5 ng/kg/day in rats) affect a small portion of the hepatocytes (up to about 10%) and appear to have little effect on either cell proliferation rates or toxicity. There may be limited mitosuppression in this region (Figure 3). Moderate doses (10–30 ng/kg/day) produce induction of between 30 and 50% of the cells. There is evidence of mitoinhibition with little enhancement.

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\text{Figure 3. Labeling index in normal hepatocytes in female Sprague-Dawley rats at the end of a 90-day initiation/promotion study. (A) The rats in this study were the initiation controls. They were treated with TCDD but not initiated with DEN. In these rats labeling was not homogeneously observed throughout the acinus. At 10, 35, and 125 ng TCDD/kg/day, labeling was reported predominantly in the periportal (pp) area. (B) In this study rats were initiated with a necrotizing dose of DEN (175 mg/kg). In these rats labeling occurred randomly (r) throughout the liver acinus. The r and pp notations at the higher doses indicate that the majority of animals in these groups had proliferation reported as r or pp. Data from Maronpot et al. (15).}
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of proliferation rates, although there is a more perportal pattern of proliferation. Finally, at higher doses (35–125 ng/kg/day) proliferation, toxicity, and the persistence of mitoinhibition combine to increase the number of risk factors for carcinogenicity. The interaction of multiple risk factors at the higher doses indicates that cancer risk should decrease disproportionately with dosage as animals move toward the low-dose range. The proliferation may be related to high-dose toxicity associated with a role of CYP1A2 induction, which leads to porphyria. Mitosuppression also seems to increase with increasing dosages. If these multiple factors interact, cancer risks should increase as a power of dose.

Based on the gene induction model, we calculated the Ah–TCDD concentration expected to be associated with a 1 in 10^6 level of induction in the centrilobular compartment (3.5 x 10^-8 M). Because the dose response for carcinogenicity is expected to be nonlinear with contributions from multiple factors, the biomarker dose–response curve at low levels of exposure would provide a conservative estimate of risk. When the biomarker falls to an expected response level of 1 per 10^5, the carcinogenic risk would be expected to be much below this level. Although molecular parameters may be poor predictors of actual risk, these markers could be used to provide a conservative bound. This more limited quantitative use of the biomarker, although perhaps disappointing from a mechanistic perspective, at least avoids low-dose extrapolation based on nothing more than policy.

**Hormesis Unraveled**

The observed production of a mitoinhibitory growth environment in the liver appears to represent an adaptive response. Although beneficial for maintenance of the correct size of the liver and probably for maintenance of the correct proportion of normal and dioxin-activated cells, this adaptive response when maintained continuously throughout the life of the animal selects for the growth of precursor lesions with mutations in critical growth regulatory genes. In this scenario, which is consistent with experiences with other tumor promoters (35), the ongoing toxicity with TCDD in the face of the homeostatic, mitoinhibited environment sets the stage for a higher probability of conversion of cells with growth regulatory lesions to more aggressive carcinoma.

Several lines of evidence support U-shaped dose–response curves for hepatic effects of TCDD, including initiation/promotion (12), carcinogenicity (2), and cell labeling (15). The downward sloping portion of these curves appears to be in the range where mitosuppression occurs. This mitoinhibition appears to protect the liver from carcinoma production. It would be misleading, however, to call this low-dose protection.

The overall U-shaped curve appears to be associated with regions of the dose–response regime in which different effects of dioxin predominate over others. At high doses the combination of proliferation, toxicity, and mitoinhibition acts to enhance carcinogenicity. At lower doses mitoinhibition, acting in the absence of toxicity and proliferative responses, appears to moderate cell proliferation and reduce the incidence of foci production and tumors relative to those in controls. This region is not a dose region where all effects of TCDD are expected to be beneficial. It is a region in which the mitoinhibition in liver predominates over other effects. In other tissues the TCDD-related effects associated with these altered cell growth characteristics may be associated with other toxic effects. One caution from this evaluation of the possibility of U-shaped curves with TCDD relates to the definitions of both hormesis and low levels of exposure. Low levels of exposure, in this context, simply refer to levels below those that have overt increases in toxic responses. The dose that causes a low-level response in one tissue may be associated with much higher levels of response in a second tissue. Hormesis is an empirical definition based on observing complex dose–response curves for specific toxic responses. When we realize that the pathogenecities of most toxic responses are composites of multiple effects of the chemical on the organ system, it is easy to see that complex curves may arise from different dose–response relationships for the contributing mechanisms of pathogenesis.

**Summary**

Molecular and biochemical biomarkers of response are qualitatively useful in assessing whether risk can be attributed to an exposure by assessing whether an increase in the biomarker has occurred. Among groups of exposed individuals, the magnitude of a biomarker in any one individual can be used to broadly categorize exposure and to rank the degree of risk for various individuals. However, it is difficult to estimate low-dose risks based solely on the intensity of biochemical and molecular markers of response. Most biomarkers are not proportionally related to the toxic response, and the underlying mechanisms of toxicity contributing to pathogenesis are themselves complexly related to dose. Therefore, it is unlikely that simple relationships will emerge between early molecular and biochemical parameters and toxic responses at low levels of exposure.

Biochemical and molecular parameters that are specific and sensitive may be useful for identifying doses below which increases in biomarker are not statistically significant. Experimental measurements alone cannot unequivocally establish a total lack of response because of the statistical power of the test systems. Biologically based pharmacodynamic models such as that for regional and cell-specific induction with TCDD have the potential to provide characterization of the functional relationships between dose and the biomarker and may increase our confidence in extending the predictions of the dose–response relationship for the biomarker to lower doses based on mechanistic considerations. In contrast to toxicity processes, molecular responses such as CYP1A family induction are simpler and depend on fewer biologic factors than toxic and carcinogenic sequelae. Therefore, these models of low-level increases in biomarkers are more likely to be testable by experimental studies than pharmacodynamic models of toxic responses. Despite difficulties in equating biomarker concentrations with specific degrees of risk, estimation of low-level risks based on these biomarkers is likely to be important in placing an upper bound on risk if it is assumed that a direct relation exists between the biomarker and toxicity.
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