Early screening of drug candidates for genotoxicity typically includes an analysis for mutagenicity in bacteria and for clastogenicity in cultured mammalian cells. In addition, in recent years, an early assessment of photogenotoxicity potential has become increasingly important. Also, for screening purposes, expert computer systems can be used to identify structural alerts.

In cases where structural alerts are identified, mutagenicity testing limited to bacteria can be conducted. The sequence of computer-aided analysis and limited testing using bacteria allows for screening of a comparatively large number of drug candidates. In contrast, considerably more resources, in terms of supplies, technical time, and the amount of a test substance needed, are required when screening for clastogenic activity in mammalian cells. In addition, the relatively large percentage of false positive results for rodent carcinogenicity associated with clastogenicity assays is of considerable concern. As a consequence, mammalian cell–based alternatives to clastogenicity assays are needed for early screening of mammalian genotoxicity. The comet assay is a relatively fast, simple, and sensitive technique for the analysis of DNA damage in mammalian cells. This assay seems especially useful for screening purposes because false positives associated with excessive toxicity appear to occur less frequently, only relatively small amounts of a test compound are needed, and certain steps of the test procedure can be automated. Therefore, the in vitro comet assay is proposed as an alternative to cytogenetic assays in early genotoxicity/photogenotoxicity screening of drug candidates.

Key Words: genotoxicity; chromosome aberration assay; micronucleus test; high-throughput.
Therefore, both European agencies (EMEA, 2002) and the U.S. Food and Drug Administration (FDA, 2000) require the photogenotoxicity testing of drugs that absorb light and penetrate into skin in relevant concentrations by systemic or by cutaneous application. Almost all of the in vitro assays used for routine genotoxicity testing have been adapted for photogenotoxicity testing by an additional treatment with ultraviolet (UV)-visible light. Methods that are used comprise cell-free investigations on naked DNA and in vitro methods, such as the photo-Ames test, the photo-mouse lymphoma mutation assay, the photo-micronucleus test (photo-MNT), and the photo-chromosomal aberration test. In addition, the comet assay has been used for photogenotoxicity testing (Brendler-Schwaab et al., 2004; Chetelat et al., 1996). Other than the UV-irradiation conditions, test methods for which Organization for Economic Co-operation and Development test guidelines are available can be regarded as validated (Brendler-Schwaab et al., 2004).

CURRENT STATUS OF CLASTOGENICITY ASSAYS

It is generally accepted that a positive response in a bacterial mutagenicity assay has implications for further drug development unless the compound is a bacteria-specific positive, for example, substances with an aromatic nitro moiety (Suter et al., 2002). A review of genotoxicity data on marketed pharmaceuticals suggested that no combination of genetic toxicity assays provided a higher predictivity of rodent carcinogenicity than the bacterial mutagenicity test alone (Snyder and Green, 2001). Therefore, a clear positive in these assays cannot be “derisked” with negative results from mammalian cell testing. In contrast, the predictive value of in vitro mammalian clastogenicity assays for rodent carcinogenicity has recently become a matter of considerable debate, with discussions directed at changing the test conditions and/or the criteria for positive calls (Kirkland et al., 2005, 2006). An analysis of genotoxicity data on 177 chemicals demonstrated that the specificity of the bacterial mutagenicity tests was 73.9%, while the corresponding value for mammalian cell mutagenicity and clastogenicity tests was below 45% (Kirkland et al., 2005). The low specificity is due, in part, to the lack of relevance of positive results for test chemicals that apparently do not react with DNA, as demonstrated by being negative in tests for mutations in bacteria, for DNA strand breaks, and/or for covalent binding to DNA (Galloway, 2000; Kirkland and Müller, 2000). In such cases, the increase in chromosome aberrations typically occur over a narrow concentration range at cytotoxic doses. In addition, indirect mechanisms (e.g., oxidative damage) may be involved. Understanding when such mechanisms are active is important in evaluating potential mutagenic hazards, since these effects are likely to be threshold dependent (Kirkland and Müller, 2000).

The high frequency of false positive clastogenic test results has resulted in recommendations to lower the limit concentration, reduce the toxicity limits that must be achieved, or to apply some “threshold” chromosome aberration response that must be exceeded (Kirkland et al., 2005). However, these approaches may also significantly reduce the sensitivity of these test systems. The limited predictivity of in vitro clastogenicity test methods for rodent carcinogenicity suggests that the present in vitro genotoxicity testing approach needs to be revise (Kirkland et al., 2006).

Potential consequences of positive in vitro clastogenicity test results are that resources (materials and time) might be wasted in trying to elucidate the nature of the positive response or that a potentially useful compound could be terminated prematurely. There are at least two approaches for solving this problem. An approach proposed by ICH S2A (1995) guideline is to evaluate the mechanism(s) underlying the positive genotoxicity results. However, such studies can be costly and exhaustive investigations require large quantities of a drug candidate which are generally not available in early development stages. Therefore, these compounds may be prematurely abandoned and candidates with a cleaner profile developed further. The second approach is improved screening for genotoxicity.

EARLY GENOTOXICITY SCREENING APPROACHES

Genetic toxicity of a drug candidate can be a reason for termination of development, and, therefore, there is a critical need for high-throughput screening methods that can predict early during discovery/development what may happen in the regulatory screening battery. Various systems have been developed that range from simplified versions of the existing bacterial and mammalian test systems to novel assays. As already discussed, it should be possible to identify many DNA-reactive carcinogens with a low incidence of false negative calls by combining an assessment for chemical structures indicative of DNA reactivity (e.g., electrophilicity) with a battery of bacterial mutational test methods (Bailey et al., 2005). For example, computer-aided structure-activity evaluations in combination with in vitro approaches have been used to assess the genotoxic potential of impurities and degradation products (Dobo et al., 2006; Muller et al., 2006). Extensive knowledge about chemical functional groups that can react with DNA and cause mutations is available in the scientific literature (e.g., Ashby and Tennant, 1991). Based on such knowledge, rule-based computer programs such as MCcase (http://www.multicase.com/products/prod01.htm), DEREK (http://www.chem.leeds.ac.uk/luk/derek/), or TOPKAT (http://www.accelrys.com/products/topkat/), among others, have been developed. However, a limitation to the approach of combining expert systems and assessment of bacterial mutagenicity are compounds with specific structural groups, such as carbamates, that are known carcinogens but not readily detected by bacterial genotoxicity tests (Allen et al., 1982). Therefore, an assessment of genotoxicity in organisms other than bacteria is an important step for early drug candidate selection. Test systems are under development that employ constructs where a promoter for
a gene induced by DNA damage is linked to a gene coding for a fluorescent protein. Using this technique, potential genotoxicity can be measured by light emission from treated cultures. However, to date, such assays have been largely restricted to bacterial or yeast systems. However, a promising new method using the Gadd45a promoter in human lymphoblastoid cells is currently undergoing validation (Hastwell et al., 2006).

If such a system proves to be robust and accurate in the prediction of carcinogenicity it may have value in early genotoxicity screening. Typically, a mammalian cell MNT is generally utilized in early genotoxicity screening of drug candidates as data from this test correlate well with results of the chromosome aberration assay (Miller et al., 1997). An advantage of the MNT over the chromosome aberration assay is that the analysis of the frequency of micronucleated cells can be automated (Frieauff et al., 1998). However, again, there are limitations that affect the biological significance of test results from this assay. Therefore, test methods that have a lower false positive rate and which allow for a higher throughput are needed. A promising candidate is the comet (single cell electrophoresis) assay, a test which has gained wide-spread use. The assay seems to be relatively insensitive to cytotoxicity as high concentrations of test compounds that clearly affect cell viability do not result in positive results (Hartmann et al., 2001). Furthermore, several steps of the procedure can be automated and low amounts of drug substance are needed to run a test. In addition, comet assay results were shown to correlate well with data from the chromosome aberration assay (Giannotti et al., 2002; Hartmann et al., 2003b). However, further comparative data are needed on compounds showing weakly positive effects or equivocal results in the chromosome aberration assay as such results are usually expected to be a consequence of excessive cytotoxicity.

COMET ASSAY: TEST SYSTEM AND STATUS OF USE IN GENOTOXICITY TESTING

The comet assay is used as for in vitro as well as in vivo studies (Tice et al., 2000). The in vitro test has various applications in hazard identification. Review articles on the comet assay procedure and its use in genetic toxicity testing include Speit and Hartmann (2000) and Tice (1995), and a general comet assay guideline has been published (Tice et al., 2000). In brief, the principle of the comet assay is based on the assessment of the migration of DNA in an agarose matrix under electrophoretic conditions. When viewed through the microscope, a cell with migrating DNA has the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments or strands migrating in the direction of the anode. Among the various versions of the comet assay, the alkaline (pH of the unwinding and electrophoresis buffer ≥ 13) method enables detection of the broadest spectrum of DNA damage and is, therefore, recommended for regulatory purposes (Tice et al., 2000). In this alkaline version, increased DNA migration is associated with increased levels of DNA damage such as strand breaks and alkali-labile sites. Furthermore, increased DNA migration is associated with the presence of single-strand breaks associated with incomplete excision repair sites. Thus, DNA excision repair can influence the comet assay in a complex way (Collins et al., 1993). While DNA repair generally reduces DNA migration by eliminating DNA lesions, ongoing excision repair may increase DNA migration due to incision-related DNA strand breaks. Thus, the contribution of excision repair to the extent of DNA migration seen in the comet assay depends on the types of induced primary DNA damage and the time point of analysis (Collins et al., 1993). Under certain conditions, the assay can also detect DNA-DNA and DNA-protein cross-linking, which in the absence of other kinds of DNA lesions appears as a relative decrease in DNA migration compared to concurrent controls. The decrease in DNA migration results from the ability of crosslinks to stabilize the DNA molecule.

One major advantage of the comet assay over cytogenetics methods is that cell proliferation is not needed for an assessment of a genotoxic potential and thus, any mammalian cell type can be used for testing. However, a limitation of the comet assay is that aneugenic effects, which recently have been given more attention as a possible mechanism for carcinogenicity (COM, 2000), and indirect DNA fidelity interaction liabilities such as effects on cell-cycle checkpoints are not detected with this assay. As discussed earlier, cytotoxicity is a confounding factor for cytogenetic test systems resulting in potential irrelevant positive effects. Similarly, a general issue with DNA damage assays, such as the comet assay, is that indirect mechanisms related to cytotoxicity may lead to enhanced strand break formation. However, since DNA damage in the comet assay is assessed at the level of individual cells, dead, or dying cells may be identified on microscopic slides by their specific image. Necrotic or apoptotic cells can result in comets with small or nonexistent head and large, diffuse tails (Olive et al., 1993) as observed in vitro upon treatment with cytotoxic, non-genotoxic compounds (Hartmann et al., 2001). However, such microscopic images are not uniquely diagnostic for apoptosis or necrosis since they may also be detected after treatment with high doses of radiation or high concentrations of strong mutagens (Rundell et al., 2003). Therefore, to avoid potential false positive effects resulting from cytotoxicity, recommendations regarding a concurrent assessment of cytotoxicity using dye viability assays have been made (Tice et al., 2000).

The comet assay can also be applied to investigate the induction of primary DNA damage in various organs/tissues of animals. The test is increasingly applied as a supplemental genotoxicity test for drug candidates to clarify the in vivo relevance of positive in vitro results (Brendler-Schwaab et al., 2005; Hartmann et al., 2004). Specific recommendations for the in vivo test with the goal of gaining more formal regulatory acceptance of the comet assay have been published following the 4th International Comet Assay Workshop (Hartmann et al.,
An updated position paper on specific aspects of test conditions and data interpretation following the International Workshops for Genotoxicity Testing in 2005 is in press (Burlinson et al., 2006) and international validation exercises have been initiated.

An important aspect of the comet assay is that it enables the assessment of the same endpoint in specific target organs or cell populations in vitro as well as under in vivo conditions. Therefore, a potential approach for more comprehensive genotoxicity testing could comprise an application of the in vitro version of the comet assay in early candidate selection followed by limited in vivo testing. Such in vivo investigations could easily be integrated into subchronic general toxicity testing commonly applied to support drug candidate selection.

MODIFICATIONS OF THE IN VITRO COMET ASSAY PROCEDURE TO ACHIEVE HIGHER THROUGHPUT

Although genotoxicity testing using the comet assay already can achieve a higher throughput compared to cytogenetic assays, the standard procedure is still not suitable for screening a large number of drug candidates per day. In addition, drug substance in early development is generally quite limited and standard cell cultures need considerable amounts of a compound. Therefore, both the drug substance amount and the rate-limiting steps of microscope slide preparation as well as analysis of cells need to be optimized. Some progress has been made by the introduction and the use of suspension cells in multiwell-chamber plates (Kiskinis et al., 2002) and automated image analysis systems (Frieauﬀ et al., 2001; Schuncka et al., 2004). However, each treated cell culture needs to be mixed with agarose, spread on individual precoated microscope slides, and be further processed as presented in Figure 1 (left panel). In addition, when using adherent cell lines for testing, individual cell cultures are usually trypsinized following the treatment with various concentrations of a drug candidate to obtain single-cell suspensions. To overcome these rate-limiting procedural steps, a modified approach is under development utilizing specific 96-well plates (multichamber plate, MCP). The development of the technique is funded by a grant of the European Union and is conducted as a collaboration between the University of Oldenburg (Germany), the company INTOX (Oldenburg), and with partners in Spain, Italy, and Portugal. In this modified technique, treated cells are maintained throughout the whole comet assay procedure on the MCP, that is, the time-consuming steps of trypsinization, transfer of cells to microscope slides, and individual processing of samples are avoided. The MCP is equipped with a speciﬁc coated surface containing agarose. Cultured cells adhere to this layer and retain their rounded morphology, which is important for computerized image localization and automated DNA damage quantification at the end of the test procedure. After treatment with compounds, cell viability is determined by staining with ﬂuorescein diacetate and evaluation by a ﬂuorescence reader. This procedure identiﬁes both cytotoxicity and genotoxicity on the level of individual cells and potential artifacts, that is, increased DNA migration as a consequence of cytotoxicity. Thereafter, the wells of the MCP are removed and the whole cell-containing layer is covered with agarose. The plate is then subjected to the subsequent steps of lysis, alkaline unwinding, electrophoresis, and staining (Fig. 1, right panel). For the assessment of DNA damage induction, a microscope equipped with a semiautomatic evaluation system is used. A laser-scanning procedure is under development that will allow for an analysis of a 96-well plate within a few minutes, enabling a real high-throughput screening for genotoxicity and for photogenotoxicity.

This modified procedure will enable the assessment of mammalian genotoxicity in a much higher throughput mode. In addition, the modiﬁed method can be performed with any adherent cell types, including such which are diﬃcult to trypsinize. An additional major advantage is, that cytotoxicity testing is integrated into the modiﬁed method (Fig. 1, right panel), with cell viability being assessed in the same cells that are used for an assessment of genotoxicity. At present, a prototype version of the MCP is used for further validation work including the evaluation of 384-well plates and the utilization of suspension cell lines. These activities will show whether the modiﬁed comet assay can be used successfully in a high-throughput fashion. An approach for early screening could consist of a basis analysis of DNA-damaging potential of a drug candidate using the comet assay. In case of a positive result, the compound could be further characterized using more standardized assays.

**FIG. 1.** Comparison of the standard and modiﬁed comet assay procedures.
SUMMARY AND CONCLUSIONS

Early screening for a genotoxic and photogenotoxic potential is vital for drug development. Genetic toxicity tests currently used include both bacterial and mammalian cell assays and are aimed at identifying all genotoxic carcinogens. Whereas a combination of expert computer systems with limited testing in bacterial mutation assays can be conducted quickly and need only minute amounts of drug substance, considerably more resources and drug amounts are needed for standard mammalian cell systems. Therefore, new methods for early in vitro mammalian genotoxicity screening are required. Test systems investigating the regulation of the human GADD45a gene or the induction of primary DNA damage, such as the comet assay, have the potential to be used as high-throughput screening assays to evaluate the genotoxic potential of drug candidates. While positive result from a bacterial test system cannot be overruled by negative results from mammalian systems, clearly negative results from the bacterial mutagenicity assay and a high-throughput mammalian screening assay would indicate a rather low risk for a genotoxic liability. In case of positive response from high-throughput mammalian assays, follow-up testing in more standard mammalian test systems might be the next step. However, considerable debate is in progress with regards to the low specificity of the established mammalian cytogenetic assays for the prediction of rodent carcinogenicity. This debate questions the biological significance of positive results obtained solely in cytogenetic assays. As a consequence, it might be more useful to perform limited in vivo testing in order to assess the in vivo relevance of the observed in vitro effects. To reduce animal use, such investigations could be integrated into subchronic general toxicity testing, which is commonly applied in the pharmaceutical industry to support the selection of drug candidates for further development.

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